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A NOTE ON THE PURIFICATION OF PICRIC ACID FOR CREATININE DETERMINATION.

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New York City.)

(Received for publication, January 24, 1929.)

In a previous communication (1) the present writer described a technique for the purification of picric acid which depends upon its recrystallization from benzene. While that process yields a satisfactory product with most samples of picric acid, occasionally some may be found which show little improvement after crystallization from benzene; hence we have been lead to develop more satisfactory procedures.

Below are described two processes, either one of which has been found to be very satisfactory even with the most impure picric acids which we have ever encountered. The one of these which uses crystallization from glacial acetic acid is especially suitable for the treatment of small amounts of material, but requires that the impure picric acid be dried before the process is used. As tested by the Folin-Doisy method (2), the product obtained reads about 12.5 to 13.5 mm. The second process, crystallization as sodium picrate, is essentially a modification of the Folin-Doisy method. We use sodium carbonate in place of the hydroxide employed by Folin and Doisy and convert the sodium salt to free picric acid directly on the filter paper. The difficulty with the Folin-Doisy process probably lies in the fact that there is more or less continuous decomposition of the picric acid through action of the strong alkali. With the substitution of carbonate we have obtained a much more satisfactory final product.

Directions for the processes follow.

1. *From Glacial Acetic Acid.*—The technical picric acid must be dried thoroughly before being used in this procedure. Dissolve 100 gm. of dry picric acid with the aid of heat in 150 cc. of

glacial acetic acid, and continue the heating until the mixture boils.¹ Pour the hot solution upon a fluted filter contained in a dry funnel which has been previously heated, and collect the filtrate in a dry beaker. Cover the beaker with a watch-glass and allow to stand for some hours, or overnight at room temperature (not in a refrigerator). At the end of this time if picric acid has not crystallized out, stir the mixture vigorously, or better, seed with a minute crystal of pure picric acid. Crystallization will begin at once and is complete within 2 hours or less. At the end of 2 hours filter with suction on a hardened filter and wash with about 35 cc. of cold glacial acetic acid. Suck as free from acetic acid as possible and dry at about 80–90°, with occasional stirring, until there is no odor of acetic acid. It is best to conduct all of these operations in a good draft of air. The yield is about 60 gm. of pure picric acid, which should read 12.5 to 13.5 mm. by the Folin-Doisy test.

2. *As Sodium Picrate.*—This procedure involves handling large volumes of solution, but permits the use of moist technical picric acid and gives a good percentage recovery of pure picric acid. The product is usually slightly better (Folin-Doisy test) than the one obtained through the use of glacial acetic acid.

It is convenient in this method to use a large porcelain enameled pail, but the enamel must be perfect at every point. Otherwise glass vessels should be used.

Place 6 liters of water in a large porcelain enameled pail and heat to boiling. Add 250 gm. of anhydrous sodium carbonate and as soon as this has dissolved add gradually (or as fast as it dissolves) 500 gm. of the moist technical picric acid. Before all of the picric acid has dissolved the mixture should be removed from the flame and stirred for a few minutes until solution of the picric acid has been effected. Filtration is usually unnecessary. The solution may be allowed to stand for some minutes and is then decanted from some dirt which has settled to the bottom. It is then allowed to stand overnight at room temperature. In the morning the crystallized sodium picrate is filtered off on a hardened filter in a large (23 cm.) Buchner funnel, with suction. The picrate on the filter is sucked dry and then washed with 2 liters of 10 per cent sodium chloride solution, and again sucked as

¹ The mixture should be heated in an Erlenmeyer flask upon an electric plate.

dry as possible. The suction is now turned off and 500 cc. of diluted (1 part of concentrated acid plus 4 parts of water) hydrochloric acid are poured on the filter and the mixture thoroughly stirred with a porcelain spatula. This acid is then sucked into the receiving flask and the process repeated with three more portions of the hydrochloric acid (a total of 2 liters of the acid being used). After the last portion of acid is sucked through, the picric acid on the filter is washed with 2 liters of cold distilled water and sucked dry. It is then removed from the filter and dried at about 90° and powdered. This product should read about 13.5 to 14 mm. by the Folin-Doisy test.

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THE USE OF MOLYBDIC ACID AS A PRECIPITANT FOR BLOOD PROTEINS.

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New York City.)

(Received for publication, January 24, 1929.)

During the many years that tungstic acid has been employed as a precipitant for blood proteins, as suggested by Folin and Wu, the method has met with practically no criticism and has probably been one of the most widely employed processes used in biochemistry. It is, however, well to have more than a single method of procedure available in connection with special problems which may arise, so we feel that in spite of the wide-spread acceptance of tungstic acid as a protein precipitant, many will find the use of molybdic acid valuable at times as an alternative procedure.

While tungstic acid has been found to leave unaffected the generally recognized non-protein constituents of the blood, we have already reported (1) that in the case of thioneine (ergothioneine) there is a marked loss following precipitation with tungstic acid. Recently Eagles and Vars (2) have reported a loss of thioneine where blood is precipitated with any of the ordinary protein precipitants.

At one time we encountered a sample of tungstate which led to no precipitation of thioneine. The J. T. Baker Company, which cooperated with us in this matter, has not been able to duplicate the particular sample which behaved so peculiarly in this respect.¹

¹ Bulmer, F. M. R., Eagles, B. A., and Hunter, G., (*J. Biol. Chem.*, **63**, 17 (1925)) reported a marked increase in the substance which interfered in the direct uric acid method (subsequently shown to be chiefly thioneine) in cases of "nickel rash," but were apparently not able to duplicate this finding in later cases of the same condition. It seems possible that during a part of their work Bulmer, Eagles, and Hunter may have been using one of the rare samples of tungstate, such as we have encountered, which does not cause loss of thioneine during the protein precipitation.

6 Molybdic Acid as a Protein Precipitant

Meanwhile, we were led to try the use of other protein precipitants in the attempt to find one which yielded blood filtrates suitable for analysis and from which there was no loss of thioneine. Among the various precipitants tried, molybdic acid was found to be by far the most satisfactory. As might be inferred, from its close relation to tungstic acid in physical properties, the precipitation of blood with molybdic acid is quite similar in technique to the familiar tungstic acid precipitation. As will be noted later on, however, there are certain definite and interesting differences. Thus we have found that if the commercial sodium molybdate is employed, there is a marked loss of thioneine during the precipitation. The same thing is true if molybdic acid dissolved in sodium carbonate is used, while if the molybdic acid is dissolved in the proper amount of sodium hydroxide, the blood protein precipitation yields filtrates which contain all of the thioneine originally present in the blood.

In technique, the use of the molybdic acid is somewhat simpler than tungstic acid since the final acid mixture, exactly as used in blood precipitation, remains unaltered for several weeks and not infrequently for many months. Following are the directions for the preparation and use of molybdic acid as a blood protein precipitant. The molybdic acid employed must be of the highest purity, usually marked as "ammonia-free" or "special." The Eimer and Amend "tested purity" grade is very satisfactory providing one is careful to note that the analysis of the product reports practically no ammonia. Eimer and Amend is apparently putting out two grades of "tested purity" molybdic acid, for one of which the term "purity" is perhaps a misnomer, as the product contains about 15 per cent of ammonia as reported in the analysis on the label. 25 gm. of pure molybdic acid and 125 cc. of normal sodium hydroxide are placed in an Erlenmeyer flask. The mixture is heated to boiling for a moment until practically all of the molybdic acid has dissolved and is then poured through a filter followed by about 100 cc. of boiling water. The filtrate is cooled and diluted to 500 cc.² The precipitating reagent is prepared by diluting 1 volume of this solution with an equal

² It may be noted that the quantity of sodium hydroxide used is not nearly sufficient to neutralize all of the molybdic acid, so that the solution obtained at this point is strongly acid.

volume of 0.4 N sulfuric acid. It is safe to prepare enough of the mixed solution to last for at least 6 weeks, and in many instances the mixed solution will keep much longer. Upon very prolonged standing, molybdic acid will finally crystallize from the solution, and where an appreciable amount of this has separated, the remaining solution becomes unfit for use. Such a condition is evidenced by failure to obtain clear protein-free filtrates. For precipitation of the blood proteins, 1 volume of blood is diluted with 7 volumes of water and then 2 volumes of the molybdic acid reagent are added, the mixture shaken, and then filtered. The use of molybdic acid as a substitute for tungstic acid was primarily developed so that thioneine determinations could be made in blood filtrates. The results in this connection are reported in detail in the paper following this one (3).

Certain other findings in relation to the use of molybdic acid as a blood protein precipitant may be summarized as follows: The rate of filtration of the precipitated blood mixture when molybdic acid is used is somewhat faster than where tungstic acid is employed. The total volume of filtrate obtained from the molybdic precipitant is also slightly greater than from tungstic acid. The acidity of the filtrates is about the same where either precipitant is used, being practically neutral in non-oxalated bloods and slightly acid where an excess of oxalate has been employed. Precipitation of the blood proteins in presence of a large excess of oxalate is usually better where molybdic acid is used than with tungstic acid in that there is less tendency for traces of protein to appear in the filtrate.

A determination of the various non-protein constituents in the filtrate from molybdic acid precipitation has shown essentially identical figures with the tungstic acid precipitation for sugar,³ creatinine, and urea. There is a slight though definite tendency for higher non-protein nitrogen figures where molybdic acid is used, and in one or two human nephritic bloods this difference has been as great as 6 to 7 mg. of nitrogen per 100 cc. of blood. In the case of pig blood which is high in thioneine content, the

³ While in most bloods there is a close agreement in sugar values whichever of the two protein precipitants is used, we have found some in which the sugar is lower after molybdic acid precipitation. This question is being further investigated.

TABLE I.

Recovery of Uric Acid Added to Blood.

All figures refer to mg. of uric acid per 100 cc. of blood.

Source of blood.	Sample No.	Protein precipitant.	Uric acid content of original blood.	Uric acid added to blood.	Uric acid found.	Added uric acid recovered.
			<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
Sheep.	1	Tungstic acid.	0	1.5	1.08	72
		“ “	0	10.0	8.00	80
		Molybdic “	0	1.5	1.39	92
		“ “	0	10.0	9.00	90
	2	Tungstic acid.	0	1.5	1.06	70
		“ “	0	10.0	8.70	87
		Molybdic “	0	1.5	1.35	90
		“ “	0	10.0	9.50	95
	3	Tungstic acid.	0	1.5	1.03	69
		“ “	0	10.0	8.30	83
		Molybdic “	0	1.5	1.30	87
		“ “	0	10.0	9.30	93
	4	Tungstic acid.	0	1.47	1.04	70
		“ “	0	9.80	8.08	82
		Molybdic “	0	1.47	1.28	87
		“ “	0	9.80	8.52	87
	5	Tungstic acid.	0	3.1	2.80	90
		Molybdic “	0	3.1	3.08	99
Human.	1	Tungstic acid.	3.64	1.5	4.68	66
		Molybdic “	3.76	1.5	4.80	72
	2	Tungstic acid.	3.20	1.46	4.36	79
		Molybdic “	3.24	1.46	4.36	76
	3	Tungstic acid.	3.64	1.66	4.72	65
		Molybdic “	3.80	1.66	4.92	67
	4	Tungstic acid.	3.40	3.44	6.96	102
		Molybdic “	3.88	3.44	7.36	101
	5	Tungstic acid.	2.30	3.68	5.24	79
		Molybdic “	2.70	3.68	5.72	81
	6	Tungstic acid.	4.48	3.88	8.24	99
		Molybdic “	5.08	3.88	8.72	96

total nitrogen figure is constantly slightly higher after molybdic than after tungstic acid precipitation.

The question of complete recovery of uric acid in blood filtrates is one that has never been definitely settled. From his latest work on this subject, Folin has concluded that one cannot be sure of better recovery than about 90 per cent (4). We have, therefore, given some special attention to comparison of uric acid recovery where the two different protein precipitants were employed.

The results are summarized in Table I.⁴

The determinations were made by the modified indirect method described by one of us (5), employing the silver lactate precipitation method described by Folin followed by the Benedict colorimetric procedure. It will be noted that when uric acid was added to sheep blood, its recovery was more complete when molybdic rather than tungstic acid was used as the protein precipitant. Where tungstic acid was employed, the percentage recovery was considerably greater for large amounts of added uric acid than for smaller quantities; thus when about 1.5 mg. of uric acid were added per 100 cc. of blood, only about 70 per cent was recovered, while with 10 mg. of uric acid per 100 cc., the recovery ranged from 80 to 83 per cent. With molybdic acid, from 87 to 95 per cent of the added uric acid was recovered in all cases. In none of these determinations was any allowance made for the trace of uric acid (0.05 to 0.1 mg. per 100 cc.) reported by Folin as being present in sheep blood.

In the case of human bloods it will be noted that there is a definite tendency to higher figures for the original uric acid content of the blood where molybdic acid is employed as the protein precipitant. There are, however, no definite differences in per cent of uric acid recovery where tungstic and molybdic acids are used for precipitation. The percentage recovery was practically the same in both cases and showed great differences in different bloods, the recoveries ranging from 65 to 100 per cent. It is interesting to note the definite differences in percentage of uric acid recovery with the different samples of human blood, the percentage of uric acid recovered depending on the blood samples and not on the precipitant employed.

⁴ For the figures in this table we are indebted to Dr. Jeanette Allen Behre.

10 Molybdic Acid as a Protein Precipitant

In conclusion it may be pointed out that, where desired, molybdic acid may be entirely substituted in place of tungstic acid as a blood protein precipitant. Molybdic acid filtrates permit the determination of thioneine as well as of all the non-protein blood constituents which may be determined in tungstic acid filtrates. It should be borne in mind that when molybdic acid is used as the blood protein precipitant, direct uric acid determinations will be very materially too high, due to the larger amount of thioneine in such filtrates. Where uric acid determinations are to be made on the filtrates from molybdic acid precipitations, the modified indirect method described by one of us (5) should be employed.

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THE OCCURRENCE AND DETERMINATION OF THIONEINE (ERGOTHIONEINE) IN HUMAN BLOOD.

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(Received for publication, January 24, 1929.)

In a previous communication (1) we reported preliminary findings concerning the determination of thioneine (ergothioneine) in blood. The present paper presents a continuation of this study and records the development of a more specific technique for thioneine determination. Molybdic acid has been adopted as a protein precipitant in place of tungstic acid which precipitates very appreciable amounts of thioneine from blood, as illustrated in Table I.¹ Detailed directions for precipitation of blood proteins with molybdic acid are contained in the paper preceding this one (2).

The technique previously described for the determination of thioneine involved precipitation of the uric acid together with the thioneine from the protein-free filtrate. The uric acid was then removed through treatment with acid sodium chloride solution and after the remaining silver salt had been dissolved in cyanide, a color determination was made as in the regular uric acid determination, and the results calculated to thioneine. The improvement in procedure presented in the present paper consists in the substitution of sodium hydroxide as the chief alkali for bringing about the color reaction in place of an excess of cyanide.

While thioneine yields good color in the presence of the uric acid reagent and hydroxide, the color given by uric acid is very much less than where cyanide is used as the alkali. Thus, where cyanide is employed, uric acid gives 7.5 times as much color as an equal weight of thioneine. With hydroxide as the alkali, uric

¹ Determinations reported in the previous paper were made upon blood filtrates prepared through heat coagulation followed by colloidal iron.

acid gives only about 65 per cent as much color as does an equal weight of thioneine. Unfortunately, however, we have to make use of an appreciable amount of cyanide to dissolve the silver salt which contains the thioneine from the blood filtrate, and this causes a relative increase in the color yield from uric acid, so that under the actual conditions of the determination, uric acid yields about 1.4 times as much color as is given by an equal weight of thioneine. This is, however, less than one-fifth as much color from the same amount of uric acid in proportion to thioneine as was obtained with the older technique, hence the specificity of the reaction is definitely increased.

The exact procedure for the determination of thioneine is as follows: 1 volume of blood is diluted with 7 volumes of water, and 2 volumes of the molybdic acid reagent described in the preceding paper are added gradually, the mixture shaken thoroughly, and filtered. 5 cc. of the clear filtrate are precipitated with 7 cc. of silver lactate solution (3), the mixture centrifuged, and the supernatant liquid poured off as completely as possible. The residue in the tube is decomposed by thorough stirring with 1 cc. of 10 per cent sodium chloride in 0.1 N hydrochloric acid, according to the Folin-Wu procedure for determination of uric acid. This mixture is centrifuged and the supernatant liquid poured off. If desired, the uric acid determination may be made in this fraction by the regular colorimetric determination (4). For the thioneine determination the residue in the tube is dissolved in a minimum amount of 5 per cent sodium cyanide which is added drop by drop with thorough stirring of the residue. It will be found that a sudden solution of the major portion of the residue takes place after the addition of the 6th drop of the cyanide solution. A small amount of undissolved material will remain but should be disregarded. 5 cc. of water are then added to the contents of the tube (or 10 cc. if the thioneine content is very high), and 0.5 cc (or 1 cc.) of the uric acid reagent and 2 cc. (or 4 cc.) of 2 percent sodium hydroxide are added. The centrifuge tube is then inverted to mix the contents and centrifuged immediately for a few seconds, after which the colored solution should be read promptly in the colorimeter against a suitable standard solution which has been prepared simultaneously. Throughout the present work pure thioneine solutions were employed as standards. On account

of the wide variation in thioneine content of various bloods, it is desirable to have three standard solutions containing respectively 0.075, 0.1, and 0.15 mg. of thioneine in 5 cc. of solution. 5 cc. of each of these standards are treated with 6 drops of 5 per cent

TABLE I.

Thioneine in Human Blood.

Results are expressed as mg. of thioneine per 100 cc. of blood.

Sample No.	Protein precipitant.	Blood thioneine.	Thioneine added to blood.	Total thioneine found	Added thioneine recovered.
		mg.	mg.	mg.	mg.
1	Tungstic acid.	2.47	3.75	4.95	2.47
	Molybdic "	7.20	3.75	11.30	4.20
2	Tungstic acid.	0	5.62	2.25	2.25
	Molybdic "	4.80	5.62	10.27	5.47
3	Tungstic acid.	0	15.00	10.80	10.80
	Molybdic "	7.65	15.00	23.85	16.20
4	Tungstic acid.	3.97	18.75	17.32	13.35
	Molybdic "	12.75	18.75	31.27	18.52
5	Tungstic acid.	2.62	28.50	23.25	20.60
	Molybdic "	7.27	28.50	37.50	30.22
6	Molybdic acid.	10.35	3.75	14.55	4.20
7	" "	6.97	7.80	15.07	8.10
8	" "	8.70	7.50	17.40	8.70
9	" "	6.22	9.75	17.17	10.95
10	" "	4.93	15.00	19.95	15.00
11	" "	7.65	15.75	23.62	15.97
12	" "	7.85	14.70	22.27	14.40
13	" "	4.95	26.25	32.85	30.32
14	" "	4.20	30.00	37.50	33.30
15	" "	10.70	7.50	17.85	7.15

cyanide, 1 cc. of uric acid reagent, and 4 cc. of 2 per cent sodium hydroxide, followed by 5 cc. of water.

In Table I is presented a summary of the results obtained upon a number of samples of human blood. For the most part these represent samples from various hospital cases and in many in-

stances several different bloods have been mixed prior to the analysis. Figures for the recovery of added thioneine are included where tungstic and molybdic acids have been employed as the protein precipitants. It will be noted that where tungstic acid is used as the precipitant, recovery of added thioneine is very incomplete and irregular, while with molybdic acid all of the added thioneine is recovered. Indeed, the tendency is toward recovery of somewhat more thioneine than has been added, a finding which we are unable to explain at present. It will be noted from the figures in Table I that the thioneine content of human blood averages about 7.5 mg. per 100 cc. with extreme variations between 4.2 and 15 mg. per 100 cc. of blood. The average figure is about half as high as the tentative figure presented in our earlier paper (1). It was suggested at that time that the figure was probably too high.

It is interesting to note that the fall in thioneine values of nearly 50 per cent is due entirely to the substitution of hydroxide for cyanide as the alkali. Since in both cases uric acid was entirely removed prior to the determination, we have here clear indication that there is in blood a third substance in addition to uric acid and thioneine which gives color with the uric acid reagent. Our work so far has not indicated what this substance may be. Studies with cystine have shown that this amino acid cannot be the source of the color reaction. According to the work of Hunter and Eagles (5), we would be led to conclude that glutathione was an important source of the color reaction. If glutathione occurs, as Hunter believes, in quantity of about 100 mg. in 100 cc. of corpuscles, it is not unlikely that it may contribute to the interference in the older cyanide technique, though our findings indicate that there must be at least one other substance in blood in addition to uric acid, thioneine, and glutathione which yields color with the uric acid reagent.

With the present technique in which hydroxide is employed as the alkali, glutathione cannot be a source of appreciable interference. After the addition of as much as 100 mg. of glutathione² per 100 cc. of blood, there is no increase in the figure obtained for thioneine.

² We are indebted to Dr. H. D. Dakin for supplying us with the sample of glutathione.

Recently, Hunter (6) has suggested a modification of the Ehrlich diazo reaction for the colorimetric determination of thioneine. If this reaction is, as Hunter believes, specific for thioneine, it would seem very probable that thioneine determinations may be made directly on the molybdic acid filtrates through employing the diazo reaction. We are at present studying this question and hope to report the results of this work in the near future.

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STUDIES ON GLUTELINS.

V. THE GLUTELINS OF RYE (*SECALE CEREALE*), AND OF BARLEY (*HORDEUM VULGARE*).

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The glutelins described in the preceding papers of this series had been previously fairly well characterized with reference to their elementary composition and the distribution of their nitrogen as determined by the Van Slyke method of analysis. Inasmuch as the method by which we prepared these glutelins (1) differs essentially from those used by other investigators, the analytical figures obtained for our preparations are not in all cases in agreement with those obtained for the previously reported glutelins. In the present paper are described the glutelins of rye and barley, proteins that have been less investigated and concerning which but little is known. Osborne (2) found that the residues remaining after exhaustively extracting rye and barley flours consecutively with salt solution and with alcohol still contained 28.3 and 41.7 per cent, respectively, of the total nitrogen of these flours. The presence of a gummy material soluble in dilute alkali interfered with the satisfactory preparation of the glutelins of these cereals.

Ritthausen (3) obtained a preparation which he called "gluten-casein," by acidifying a dilute alkaline extract of rye flour with acetic acid. The same author also obtained a preparation which separated on cooling from a hot alcoholic extract of barley flour, which he assumed was similar to the gluten-casein obtained from rye. In view of the method by which he prepared this product, however, it is very doubtful that he was dealing with a glutelin.

Einhof (4) and von Bibra (5) separated from rye and barley glutelins of questionable purity.

While our investigation on the rye and barleyutelins was in progress, a paper by Larmour (6) appeared, in which he described the preparation and analyses of theutelins of these two cereals. The ryeutelin was prepared by a method similar to that used by Osborne. The slightly different method used for preparing the barleyutelin resembled Fleurent's alcohol-alkali method for the preparation ofutelins.

By using our ammonium sulfate method for precipitatingutelins, we were able to separate twoutelins from barley. The α -utelin separated from the alkaline medium by addition of enough ammonium sulfate to make the solution 1 to 3 per cent saturated. The β -utelin separated at 18 per cent of saturation. Rye flour was found to contain only oneutelin, which separated at 3 per cent of saturation with ammonium sulfate.

Preparation of Rye Utelin.

A quantity of rye flour (500 gm.), milled in the laboratory and separated from the bran, was mixed with 4 liters of 0.2 per cent NaOH in 50 per cent alcohol. The mixture was stirred for 5 hours with a mechanical stirrer and then allowed to stand overnight. The supernatant liquid was decanted, centrifuged, and then filtered through paper pulp. Theutelin was precipitated from the clear filtrate by addition of enough HCl to bring the solution to pH 6.8. After the precipitate had settled, as much as possible of the supernatant liquid was siphoned off, and the remainder was removed by centrifugalization. Theutelin precipitate was redissolved in alcoholic alkali and reprecipitated by HCl as described above. The precipitate was then washed twice with distilled water containing a little HCl. The purpose of this washing was to remove the alcohol. The precipitate was readily dissolved in 500 cc. of aqueous 0.2 per cent NaOH, and to the clear liquid enough saturated ammonium sulfate solution was added to make the mixture 5 per cent saturated with that salt. The ryeutelin separates at about 3 per cent of saturation. The excess of ammonium sulfate was added to assure complete separation of theutelin. After standing for about 1 hour the precipitate was separated by centrifugalization and washed twice with a 5 per cent saturated solution of ammonium sulfate, and then washed with distilled water containing enough

HCl to bring the pH to 6.8. The washing was continued with 70 per cent alcohol, and the glutelin was finally dehydrated with

TABLE I.

Elementary Composition of the Glutelin of Rye and of the α -Glutelin of Barley in Percentages of Moisture- and Ash-Free Protein.

	Glutelin of rye.	α -Glutelin of barley.
	per cent	per cent
Carbon.....	53.05	54.31
Hydrogen.....	6.35	6.94
Sulfur*.....	1.12	1.21
Nitrogen.....	16.72	16.16
Ash.....	0.454	0.408

* Sulfur was determined by the peroxide method by using Parr's heat ignition bomb (7).

TABLE II.

Distribution of Nitrogen in the Glutelin of Rye and in the α -Glutelin of Barley as Determined by the Van Slyke Method, Corrected for Solubility of Bases.

	Glute- lin of rye.	α -Glu- telin of barley.	Amino acids expressed in percentages of the ash- and moisture-free proteins.		
				Glute- lin of rye.	α -Glu- telin of barley.
	per cent	per cent		per cent	per cent
Amide N.....	10.87	16.09	Cystine.....	2.56	3.10
Humin N.....	1.62	2.52	Arginine.....	7.07	5.59
Cystine N.....	1.79	2.24	Histidine.....	2.75	1.09
Arginine N.....	13.61	11.13	Lysine	5.39	2.88
Histidine N.....	4.46	1.84			
Lysine N.....	6.18	3.41			
Amino N in filtrate from bases.....	55.53	52.79			
Non-amino N in filtrate from bases.....	4.78	9.15			

absolute alcohol and ether in the usual manner. The yield was about 0.7 per cent, based on the flour used.

Rye flour was found to contain only one glutelin. The precipitation range of the glutelin with ammonium sulfate corresponded

to that of an α -glutelin. The filtrate obtained from the rye glutelin showed the presence of another protein, which precipitated at 32 per cent of ammonium sulfate saturation. The high per cent of saturation with ammonium sulfate at which it separated indicates that this fraction was a globulin.

Preparation of Barley Glutelin.

The method used for the preparation of the barley glutelins was similar to that described above for rye glutelin. Barley flour, however, was found to contain two glutelins. The α -glutelin separated at 1 to 3 per cent of saturation with ammonium sulfate, and the β -glutelin at 18 per cent of saturation. The amount of β -glutelin is rather small.

The elementary composition of the rye glutelin and of the α -glutelin of barley is given in Table I. The amino acid composition of both these glutelins resembles fairly closely that of the α -glutelin of wheat. The percentages of amide N, arginine N, and non-amino N in the filtrate from the bases (Table II) for the α -glutelin of barley resemble those found for wheat glutelin.

The characteristic property of wheat flour of forming a dough, a property not shared by rye or barley flour, apparently does not depend on the chemical composition of its glutelins.

The isoelectric point (8) of the rye glutelin was found to be at pH 6.2, and that of the α -glutelin of barley at pH 6.4.

SUMMARY.

Rye (*Serale cereale*) contains one glutelin which separates from an aqueous 0.2 per cent sodium hydroxide solution by addition of ammonium sulfate to 3 per cent of saturation. Barley (*Hordeum vulgare*) contains two glutelins, which separate from the alkaline medium at 1 to 2 per cent, and at 18 per cent, respectively, of saturation with ammonium sulfate.

The distribution of the nitrogen according to the Van Slyke method, and the elementary composition of the rye glutelin and the α -glutelin of barley were determined.

The isoelectric point of rye glutelin was found to be at pH 6.2, and that of the α -glutelin of barley at pH 6.4.

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THE DETERMINATION OF LACTIC ACID.*

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Lactic acid is a substance of major significance throughout the orders of life, from microbe to man. Its relation to the carbohydrate metabolism has been very extensively studied; and it is not surprising, therefore, that many methods have been proposed for its quantitative estimation. Most of the methods depend upon the conversion of lactic acid into acetaldehyde (1, 2), the latter being determined as such, or colorimetrically after reacting with some color-producing substance. Of these methods, perhaps the most precise is that of Friedemann, Cotonio, and Shaffer (3, 4). Lactic acid, according to this method, is oxidized by acid KMnO_4 in the presence of MnSO_4 . The resulting acetaldehyde is aerated out of the solution, absorbed in bisulfite, and determined by the Clausen (2) titration method. The chief advantages of the method over the older oxidation methods of von Fürth and Charnas and Clausen are (1) its speed, (2) the considerably increased yield of acetaldehyde, and (3) the smaller fluctuations between individual determinations. This method, as in the case of the older methods, determines also the more volatile sulfite-binding substances which may be produced from many other compounds besides lactic acid. Their effect on the determination may be minimized, however, as was shown by Friedemann, Cotonio, and Shaffer, merely by allowing the vapors to pass upward through a cooled reflux condenser, the less volatile substances being condensed with the steam and returned to the solution where apparently they are destroyed by further oxidation.

* A preliminary report of this work was presented as a demonstration at the meeting of the Society of Biological Chemists, April, 1928. (Kendall, A. I., and Friedemann, T. E., *J. Biol. Chem.*, **78**, p. lxi (1928).)

In actual practice the materials analyzed for lactic acid contain relatively very large quantities of interfering substances, many of which may also yield bisulfite-binding substances when oxidized. In the case of blood and tissues the chief interfering substances are proteins and carbohydrates. The former may be removed by tungstic acid or trichloroacetic acid and the latter may be removed by treatment with $\text{CuSO}_4\text{-Ca(OH)}_2$.¹ The resulting filtrate contains relatively small amounts of nitrogenous substances, and gives fairly concordant results either by the method of Clausen or the method of Friedemann, Cotonio, and Shaffer.

Such simple procedures for removing interfering nitrogenous substances do not apply to many of the other materials which are often encountered in biochemical work. Examples of such are bacterial culture media and urine. The materials mentioned contain very large amounts of non-protein substances. The various mercury reagents, especially acid mercuric nitrate, which are very efficient in removing even the very simple nitrogenous compounds may be used, if care is taken in deproteinizing blood; they are, however, entirely unsuited for the preliminary preparation of culture media or urine. The results for lactic acid are invariably low. Even by the most careful neutralization with sodium bicarbonate, as suggested by Ronzoni and Wallen-Lawrence (4), or with BaCO_3 , only about 85 to 95 per cent of the added lactic acid may be recovered. The results are low even with a pure lactic acid standard, with the amount of mercuric reagent necessary for the complete removal of nitrogen from peptone media.

Since the usual precipitants are unsuitable the logical procedure would seem to be extraction by ether. But this is open to some criticism. In addition to the possibility of incomplete extraction there is the possibility also of losing some of the lactic acid which has already been extracted. Known small amounts of lactic acid, for example, often are incompletely recovered if added to ether which has been refluxed for some time and the mixture then evaporated in accordance with the directions of Clausen. Considerable varia-

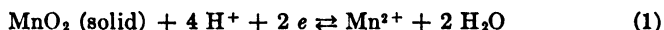
¹ The $\text{CuSO}_4\text{-Ca(OH)}_2$ procedure removes many other substances besides the sugars. Examples of such are tartaric, citric, glyceric, and many of the higher saccharinic acids, and polyatomic alcohols. Some, like tartaric acid, can be removed completely. A large excess of Ca(OH)_2 appears to be essential.

tions are also noted when peptone culture media are extracted by ether and evaporated as before. These effects are possibly due to oxidation by the peroxides which are formed from the ether.

The experience gained in this laboratory indicates that there is at present no satisfactory procedure which will yield reasonably reliable results on such complex materials as culture media or urine. The results are probably too high even in the case of blood (which represents a fairly simple mixture) where it is possible to remove most of the nitrogenous and other interfering material, for, as will be shown, by slightly changing the conditions of the oxidations, the lactic acid values in some instances may be reduced by as much as 10 to 30 per cent.

Although it may be impossible in some instances by present methods to determine the absolute amount, it is often desirable to measure merely the *change* in lactic acid content, *i.e.* a gain or a loss in a given solution or medium. By making certain changes in the procedure the interfering effect of the nitrogenous materials may be greatly diminished and, although the lactic acid values obtained by direct oxidation may be slightly higher than those obtained from the ether extract, the results so obtained are believed to be more reliable as a measure of the changes in the lactic acid content than can be determined by any of the present methods. One may thus, for example, follow the changes which occur in a culture medium during the growth of microorganisms.

In studying the oxidation of pure lactic acid solutions it was found that a control of at least four factors is essential for the highest yield; namely, (1) the concentration of the oxidizing agent added, (2) the acidity of the solution, (3) the concentration of MnSO_4 , and (4) the amount of lactic acid oxidized. (1), (2), and (3) would appear to follow from the equation



The maximum yield of acetaldehyde is obtained when the lactic acid is oxidized by a very dilute solution of KMnO_4 or MnO_2 in the presence of very large quantities of MnSO_4 and at a relatively low acid concentration. *In general, therefore, those conditions which appear to reduce the oxidation potential also reduce the overoxidation and coincidentally increase the yield of acetaldehyde.*

Phosphoric acid was found to be the most suitable acid, and the

optimum acidity appears to be 0.03 to 0.15 M. Although KMnO_4 may be used for most oxidations, it cannot be used where the amount of interfering oxidizable material is relatively large. For such oxidations the use of colloidal MnO_2 is particularly recommended. If added drop by drop a strong colloidal MnO_2 suspension may be added without causing much overoxidation, as is the case when KMnO_4 is used.

The procedure which will be described is essentially the same as that of Friedemann, Cotonio, and Shaffer. However, a number of changes have been made which increase the precision. Certain details of the older procedure require further emphasis, for it is found that the results are more precise and less subject to slight variations only when these details are followed. For this reason a rather detailed description of the procedure will be made.

Description of Method.

Apparatus.

The apparatus is the same as that described by Friedemann, Cotonio, and Shaffer. A few precautions are essential for success. First of all, an efficient, well cooled condenser is necessary to insure consistent results. The Hopkins type seems to be the best suited for this purpose. This should have not more than 3 mm. of space between the inside condenser tube and the outside tube, and the height of the condensing surface should be at least 25 cm. With this apparatus all the water vapor is condensed at a point below the tube through which the acetaldehyde passes to the absorption tower, and none is lost through solution in the vapors which are collected beyond.

Solutions.

1. *Oxidizing Agent.*—Either KMnO_4 or colloidal MnO_2 may be used. Colloidal MnO_2 is prepared as follows:

Method A.—9 gm. of glucose, dissolved in 1 liter of approximately 0.5 N NaOH, are added to 3 to 4 liters of a solution of KMnO_4 (53 gm.).² The mixture is warmed to 70° and kept at this

² The oxidation of glucose by alkaline KMnO_4 yields almost 2 molecules of oxalic acid and slightly more than 2 molecules of CO_2 : $\text{C}_6\text{H}_{12}\text{O}_6 + 10 \text{O} + 20 \text{e} \rightarrow 2 \text{CO}_2 + 2 \text{C}_2\text{H}_2\text{O}_4 + 4 \text{H}_2\text{O}$. Since $\text{MnO}_4^- + 4 \text{H}^+ + 3 \text{e} \rightarrow \text{MnO}_2$ (solid) + 2 H_2O , it can be seen that $\frac{20}{3}$, or 6.7 equivalents of KMnO_4 are required.

temperature for 15 minutes. If the color disappears during this period, saturated aqueous KMnO_4 is added until an excess remains. The solution is then cooled, filtered through a large Buchner funnel, and washed well with water. The precipitate of MnO_2 , which is granular at first, becomes more and more dispersed with the removal of alkali and salts. To facilitate the removal of salts, the precipitate is thoroughly broken up in a small amount of water (an egg beater is very effective for this purpose) and again returned to the Buchner funnel and washed. This salt-free precipitate is finally broken up thoroughly in water and diluted to 2 to 4 liters. The coarser particles settle out after a few hours. The upper two-thirds of the suspension, which is practically free from visible particles, is removed by suction after standing 12 to 24 hours. The remainder, containing coarser particles, is again suspended, agitated, and allowed to settle. This may be repeated several times. The supernatant, colloidal suspensions are united and adjusted to about 0.05 N or 0.1 N strength, in the following manner. A known amount of this colloidal suspension is run into a solution of KI acidified with H_2SO_4 , and titrated with standard thiosulfate.

$$1 \text{ cc. } 0.1 \text{ N thiosulfate} = 1 \text{ cc. } 0.1 \text{ N colloidal } \text{MnO}_2.$$

Solutions prepared in this manner are not entirely colloidal, although no particles can be seen in them. Upon standing, a sediment settles out. This sediment is not granular, however, and can be readily resuspended. Electrolytes precipitate it.

The yield of this colloidal MnO_2 is not large; only about 50 to 70 per cent of the theoretical quantity is obtained when the precipitate is suspended and worked over five times. Additional treatment increases this yield somewhat.

Any substance which is readily oxidized by alkaline permanganate may be substituted for glucose in this preparation.

Method B.—A fairly stable and satisfactory suspension of MnO_2 may also be prepared by allowing KMnO_4 to react with MnSO_4 . A concentrated solution of MnSO_4 , containing slightly more than 3 equivalents, is run, with stirring, into a solution of KMnO_4 (2 equivalents). The resulting granular precipitate of MnO_2 is washed free of salts, *etc.*, as in Method A above.

Method C.—A fairly satisfactory dilute suspension for use in the analysis of blood and tissue extracts, or in any sample which con-

tains relatively small amounts of other oxidizable materials besides lactic acid, may be prepared by adding a dilute MnSO_4 solution drop by drop with shaking to a dilute (0.01 N) KMnO_4 solution until the color is almost discharged.

0.01 N to 0.005 N permanganate or colloidal MnO_2 is recommended for the determination of from 1 to 15 mg. of lactic acid. For smaller amounts of lactic acid, more dilute solutions may be used. However, if large amounts of other oxidizable materials are also present a strong colloidal MnO_2 suspension should be used.

2. *Phosphoric Acid*.—A 2 M solution is prepared by diluting 135 cc. of the syrupy acid (85 per cent, 15 M) to 1 liter.

3. *Manganese Sulfate*.—A 10 per cent solution of $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ (the ordinary salt) is used.

4. *Talcum*.—Finely powdered.

5. *Sodium Bisulfite*.—1 per cent solution. 5 to 10 cc. (more for large amounts of lactic acid) are used for each determination. Enough water is added to cover the beads in the tower. There should be an excess of bisulfite, equivalent to 5 cc. or more of 0.1 N iodine, over the amount required to unite with the aldehyde.

6. *Starch Indicator*.—5 gm. of arrowroot starch are suspended in 10 to 20 cc. of cold water and poured into 500 cc. of boiling water. Boiling is continued for 20 minutes. The flask is covered with a beaker and cooled. The supernatant clear solution is used. If care is taken to avoid contamination, this solution will keep for several weeks. If the solution becomes contaminated, the end-point with very dilute iodine (0.002 N) is pink instead of blue.

7. *Alkali to Liberate Bound Bisulfite*.—Saturated NaHCO_3 solution.

8. *Standard Iodine*.—0.1 N iodine. This must be standardized frequently against thiosulfate which in turn must be standardized occasionally against $\text{KH}(\text{IO}_3)_2$ or KIO_3 . The 0.1 N iodine is diluted each day to 0.01 or 0.002 N strength and should be protected from direct sunlight. Each cc. of the 0.01 N iodine solution is equivalent to 0.45 mg. of lactic acid.

9. *d,l-Zinc Lactate Standard*, $\text{Zn}(\text{C}_3\text{H}_5\text{O}_3)_2 \cdot 3 \text{H}_2\text{O}$.—U. S. P. lactic acid is boiled with an excess of c. p. zinc carbonate and filtered hot. The solution is evaporated to a small volume and allowed to crystallize at room temperature. The crystals are transferred to a Buchner funnel and washed several times with

small portions of ice-cold water. The product is twice recrystallized. The mother liquors, which contain some *d,l*- and much *d*- or *l*-lactate, are discarded (5, 6). Its purity may be determined either by a moisture determination (24 hours at 130°) or by the determination of zinc as ZnO by ignition to constant weight.

Procedure.

The solution containing lactic acid is placed in a 300 cc. Kjeldahl flask. If the solution is strongly acid or alkaline (greater than 0.05 *N*) or contains much buffer, the reaction is adjusted approximately to neutrality (phenol red) by adding either NaOH or H₂SO₄. The indicator may be added directly to the solution. Alcoholic solutions of indicators must not be used. From 1.5 to 8 cc. of H₃PO₄, depending upon the total volume of the solution in the flask, 10 cc. of MnSO₄ solution and a pinch of talcum are next introduced. Sufficient water is added to bring the total volume to 50 or 100 cc. A final acidity of 0.06 to 0.10 *M* H₃PO₄, at least 1 per cent MnSO₄, and a total volume of 50 cc. are preferable. The reagents, especially the MnSO₄, should be rather carefully measured.

The flask is now connected to the condenser, and the suction is started.³ The solution is brought to boiling, and then the MnO₂ (or KMnO₄) is allowed to drop in, but only after the vapors are condensing in the reflux condenser. The oxidizing agent is added drop by drop at a rate of not more than 1 or 2 per second until an excess has accumulated in the flask. When colloidal MnO₂ is used, an actual excess should be present for at least 10 minutes. If the solution becomes decolorized, more MnO₂ must be added. The addition of the oxidizing agent requires up to 10 minutes. A total of 20 minutes aeration is sufficient, time being counted from the time the oxidation is started.

When the aeration is finished, the flame is removed, the suction is stopped, and the tower is washed with water. Usually five 5 to 10 cc. portions of water suffice. The total volume should be

³ If the solution contains acetone, acetoacetic acid, or other volatile bisulfite-binding substance, it is boiled for 5 minutes while aerating with an empty flask attached to the tower.

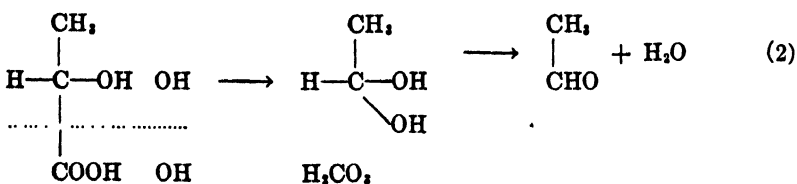
kept as small as possible, because the titration can be carried out more rapidly and the end-point is sharper under these conditions. The excess bisulfite is removed with 0.1 N iodine, starch (1 cc.) being used as an indicator. The iodine is added until it is present in slight excess. This excess in turn is removed by 1 drop of 0.1 N thiosulfate solution. The end-point is finally adjusted with the dilute iodine solution in such a manner that 1 drop of this dilute iodine solution produces a clearly discernible *change*, but not a deep blue, in the colorless solution. The bound bisulfite is then liberated by adding 5 to 10 cc. of the saturated NaHCO_3 solution (2) and is titrated with the dilute iodine solution (0.01 or 0.002 N). Here again the end-point to be attained is the *first change* from the colorless. This should persist for 30 seconds.

Frequent blanks should be run on the reagents and the blank titration is subtracted as a correction before calculating the results.

The yield in pure solutions of lactic acid is from 97 to 99 per cent, *depending upon the conditions*. Correction for the yield is not ordinarily made when blood, muscle, culture media, *etc.*, are analyzed because of the presence of other compounds which also yield bisulfite-binding substances. However, if the amount of such interfering substances is relatively small and if accurate results are desired, the results may be multiplied by a factor which is obtained by analyzing similar solutions containing known amounts of zinc or lithium lactate. The factor thus obtained should not be used in subsequent analyses of lactic acid containing solutions of unknown composition unless the concentration of reagents and conditions are the same.

Factors Which Influence the Oxidation.

Oxidizing Agent.—The oxidation of lactic acid to acetaldehyde, which apparently proceeds according to the following reaction



may be accomplished by many oxidizing agents.⁴ The quantitative conversion into acetaldehyde, however, seems to be effected by only a few oxidizing agents. So far KMnO_4 has been found most satisfactory and the results are most reliable when a large amount of MnSO_4 is present (3).

According to Friedemann, Cotonio, and Shaffer, the oxidation of the lactic acid is really due to MnO_2 , and not KMnO_4 . This is based upon the well known fact that KMnO_4 is decolorized and MnO_2 is precipitated when KMnO_4 is added to an acid solution of MnSO_4 . The oxidation-reduction potential also drops until it is approximately equal to that of an MnO_2 suspension.



"In this reaction by an exchange of electrons, the permanganate (Mn^{7+}) is transformed to a lower intensity level (Mn^{4+}), which is still high enough to oxidize the lactic acid, but not so intense as to oxidize rapidly the acetaldehyde" (3). If MnO_2 is the effective oxidizing agent it follows that it should be possible to oxidize lactic acid to acetaldehyde to the same extent, or better, with MnO_2 as with $\text{KMnO}_4 + \text{MnSO}_4$. Boas (7), in 1893, used ordinary black oxide of manganese (MnO_2), obtaining a very good yield of acetaldehyde. The oxidation rate with the latter is slow. Hydrated or colloidal MnO_2 , however, prepared as above, is very satisfactory. The reagent can be added more rapidly, the fluctuations in the results are smaller, and the yield is somewhat higher than with KMnO_4 .

The oxidation appears to depend upon the physical state of the manganese dioxide. Ordinary very finely powdered MnO_2 oxidizes lactic acid incompletely within a reasonable time, due perhaps to the slow rate of oxidation. The oxidation is very rapid and the yield is greater than 97 per cent (depending upon the conditions) if precipitated (hydrated) MnO_2 is used. The precipitated MnO_2 remains granular if not washed as thoroughly as described in the directions above. Such granular MnO_2 may be added all at once

⁴ Acetaldehyde may be obtained by the oxidation with KMnO_4 or MnO_2 of many substances having the general formulas $\text{CH}_3\text{—CHOH—CO—R}$ or $\text{CH}_3\text{—CHOH—CHOH—R}$, but apparently not $\text{CH}_3\text{—CHOH—CH}_2\text{—R}$. β -Hydroxybutyric acid yields practically no acetaldehyde. Rhamnose, propyleneglycol, and 1,2-dihydroxybutyric acid, on the other hand, are examples of substances which yield acetaldehyde. 1,2-Dihydroxybutyric acid yields acetaldehyde almost quantitatively (6).

without very greatly lowering the yield.⁵ The colloidal material must be added more slowly.

The results of adding 0.1 N MnO_2 to varying amounts of lactic acid are shown in Chart I. The acidity and MnSO_4 concentration were kept constant. The yield of acetaldehyde decreases as the amount of substance analyzed becomes smaller. It seems to be a function of the logarithm of the lactic acid concentration, the effect being most pronounced when small amounts

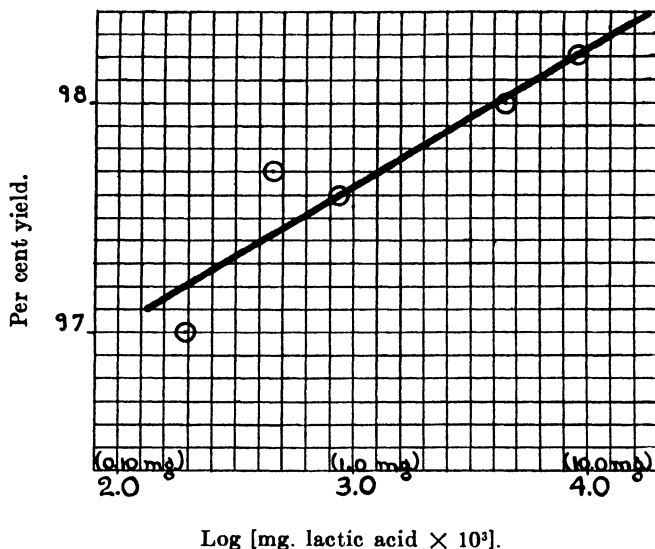


CHART I. Yield of acetaldehyde from varying amounts of lactic acid.

of lactic acid are analyzed and diminishing as the amount oxidized increases.

The unfavorable effect of strong MnO_2 on the yield (Chart I) from small amounts of lactic acid may be overcome by using more dilute MnO_2 . This is shown in Chart II, the acidity and MnSO_4 being kept constant as before. Three analyses were made at each concentration and all results were used to obtain the average. The yield from 0.45 mg. of lactic acid increases from 98.1 per cent to

⁵ The use of the granular hydrated MnO_2 for various reasons is not recommended. The colloidal preparations have proved far more satisfactory.

99.1 per cent in a regular progression as the concentration of added MnO_2 is decreased from 0.1 N to 0.005 N. It is therefore advisable to use very dilute oxidizing agent when small amounts of lactic acid are analyzed. This is not so important when larger quantities are present, and in this case the more concentrated MnO_2 may be used.

Manganese Sulfate.—It has been pointed out that probably one of the chief functions of manganese sulfate, in the oxidation of lactic acid by permanganate, is to reduce the permanganate rapidly. Permanganate has a high oxidation intensity, and by its presence, even if momentary, reduces the yield of acetaldehyde, presumably

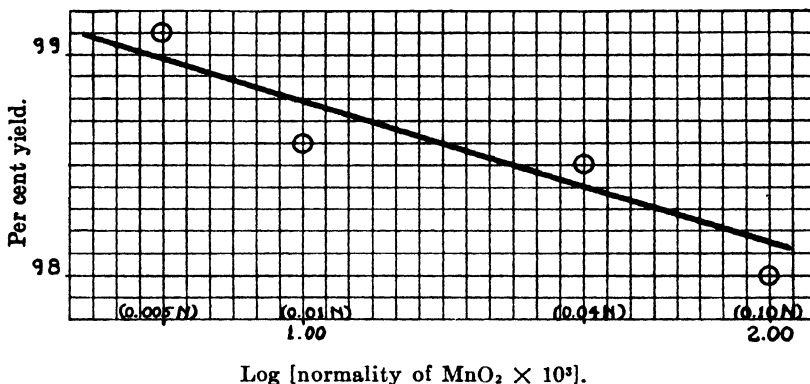


CHART II. Effect of concentration of the MnO_2 solution used for oxidation on the yield of acetaldehyde from 0.45 mg. of lactic acid, the total acidity and MnSO_4 concentration being the same in all.

by further oxidation of some of the acetaldehyde formed from the lactic acid.

From Equation 3 above, it is apparent that the concentration of MnSO_4 should greatly influence the reduction to Mn^{4+} . The rate of removal of Mn^{7+} should be accelerated as the concentration of Mn^{2+} is increased. Overoxidation, due to the presence of KMnO_4 , would therefore tend to be diminished as the rate of its removal is increased. Thus, in the presence of a very large amount of MnSO_4 , good yields may be obtained even with 0.1 N KMnO_4 .⁶

⁶ This is clearly shown by the analysis with 0.1 N KMnO_4 made by Friedemann, Cotonio, and Shaffer (3) (Table IV, p. 354). From their results it can be seen that the yield of acetaldehyde increases regularly as the MnSO_4 concentration is increased.

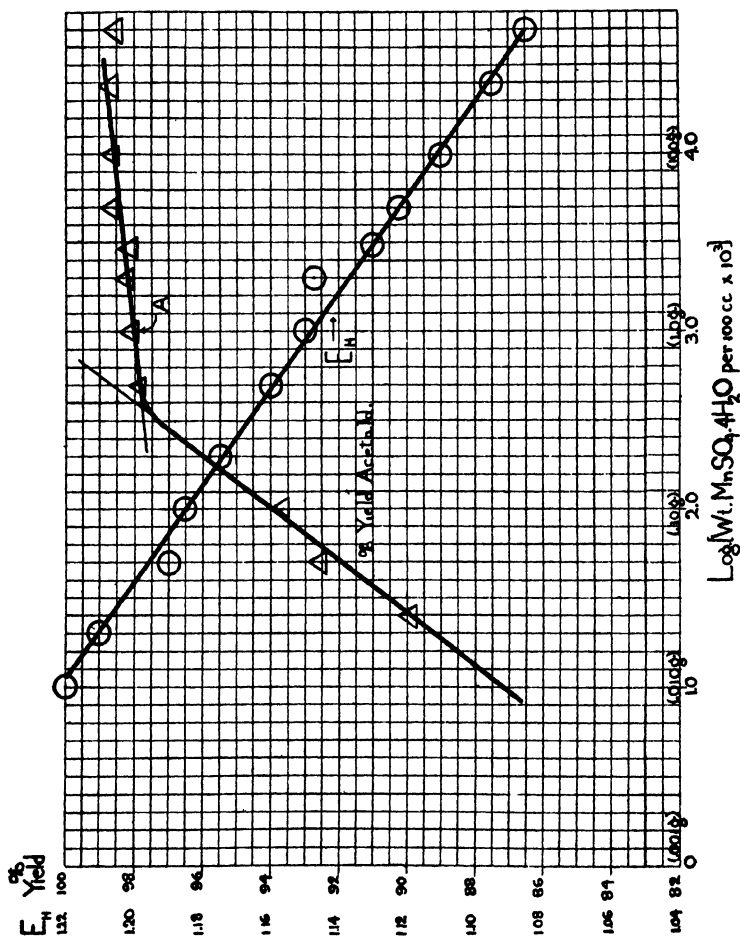


CHART III. Effect of changing the concentration of manganese sulfate on the yield of acet-aldehyde. The effect on the oxidation-reduction potential is also shown.

But the MnSO_4 has still another function. It affects the action of the MnO_2 either added as such or as it is formed from KMnO_4 . This would follow from Equation 1. The effect of Mn^{2+} can be directly demonstrated by measurement of the electrical potential of known solutions against a platinum electrode. If a small amount of MnSO_4 is added to a solution of KMnO_4 in $N \text{ H}_2\text{SO}_4$, it is found that the potential drops from about 1.63 volts (against the normal hydrogen electrode) to about 1.35 volts, which is the potential usually obtained from a suspension of MnO_2 . Upon further addition of MnSO_4 the potential continues to fall at a regular rate, depending upon the concentration of MnSO_4 present.

The effect of changing the manganese sulfate concentration on both the oxidation-reduction potential and the yield of acetaldehyde from the oxidation of known amounts of lactic acid is shown in Chart III. The same reagents were used in obtaining both series of results. 4 cc. of 1.67 $M \text{ H}_3\text{PO}_4$, a measured quantity of a 10 per cent solution of MnSO_4 , and enough water to bring the volume to 95 cc. were mixed in a flask. 5 cc. of 0.1 N colloidal MnO_2 were added to all of the flasks. Solid MnSO_4 , instead of the 10 per cent solution, was added to the last three flasks which contained 10, 25, and 50 gm., respectively. With the acidity and MnO_2 concentration thus kept constant, and only the MnSO_4 concentration varied, the results of potential measurements show, as would be expected, that the oxidation-reduction potential varies as the logarithm of the MnSO_4 concentration. By appropriate calculation it can be shown that these results are in accord with the equation

$$E_R = E_0 - \frac{RT}{2F} \ln \frac{[\text{Mn}^{2+}]}{C}$$

in which C is the concentration of Mn^{4+} , which is assumed to be constant in all.

Since there are no sharp breaks in the curve it may be assumed that MnO_2 (not necessarily the lower oxidation stage, Mn_2O_3) is the effective oxidizing agent and that MnSO_4 decreases the tendency to change from Mn^{4+} to Mn^{2+} . In other words, the function of the Mn^{2+} is to reduce the oxidizing intensity of the MnO_2 .

Such a reduction in oxidizing intensity, as the result of the

presence of Mn^{2+} , should be reflected in the yield of acetaldehyde from lactic acid. As can be seen (Chart III), the yield increases regularly as the concentration of manganese sulfate increases. The point A on the chart shows the recovery when 1 gm. (or 10 cc. of 10 per cent MnSO_4) is present in 100 cc., and represents the conditions described in the procedure above (p. 29). The yield may be increased slightly by adding larger amounts of MnSO_4 than are suggested in the procedure. For practical purposes, however, this quantity is sufficient.

Effect of Acidity.—Although the yield of acetaldehyde is affected by the concentration of the MnO_2 dropped in and the MnSO_4

TABLE I.

Effect of Acidity on Yield of Acetaldehyde.

10 cc. of 10 per cent MnSO_4 ; total volume, 100 cc.; 0.1 N MnO_2 dropped in. 9.0 gm. of lactic acid were oxidized.

Acid or buffer mixture.	Molar concentration.	No. of determinations.	Average per cent yield.
H_2SO_4	0.5	6	93.0
H_3PO_4	0.333	9	95.6
H_3PO_4	0.167	6	97.8
H_3PO_4	0.083	9	97.8
H_3PO_4	0.067	20	97.8
H_3PO_4	0.042	3	97.7
$\text{H}_3\text{PO}_4\text{-KH}_2\text{PO}_4$	0.20 (pH 2.5—3.0)	3	96.8
KH_2PO_4	0.20	3	80.5
H_3BO_3	2 per cent.	6	28.8

present in solution, it is not so markedly affected, within rather wide limits, by changes of the acidity of the solution. In Table I are shown the results of oxidations carried out at various concentrations of phosphoric acid. As can be seen, the results are lowest at high acidity. They increase as the acidity is decreased, reaching a maximum and remaining quite constant over a range of acidity from 0.167 to 0.042 M. It is almost as high in 0.2 M buffer mixtures of $\text{H}_3\text{PO}_4\text{-KH}_2\text{PO}_4$, pH 2.5 to 3.0. However, a large diminution is noted, and the fluctuations become more pronounced, when the oxidations are carried out in solutions of lower hydrogen ion concentration. In H_3BO_3 , for example, the minimum yield

obtained by 30 minutes oxidation and aeration was 17.2 per cent, while the maximum was only 66.5 per cent.

The oxidation, therefore, appears to depend somewhat upon the hydrogen ion concentration of the solution. It is not peculiar only to H_3PO_4 . The same results may be obtained with H_2SO_4 , provided the acidity is about the same as that of H_3PO_4 . However, on account of its high degree of dissociation, the maximum yield with H_2SO_4 is obtained over a much more limited range of total acid concentration. Acetic acid, on account of its volatility and limited dissociation, is not a suitable acid; also, the presence of previously unneutralized alkali in the lactic acid solution may bring the hydrogen ion concentration into a range where the results may be low and variable. Phosphoric acid, therefore, on the whole appears to be best suited for the purpose. It is non-volatile and has a sufficiently low dissociation constant so that considerable variation in the amounts of this acid may be employed without greatly changing the hydrogen ion concentration.

It is interesting in this connection to compare the changes in oxidation-reduction potential with the results shown in Table I. According to Equation 1 the reduction of Mn^{4+} to Mn^{2+} consists essentially of the reaction of Mn^{4+} with 4 H^+ and 2 e . The influence of the hydrogen ion concentration is shown by the following measurements

The solutions were the same as were used in the lactic acid determinations. Water, 10 cc. of 10 per cent manganese sulfate solution, 5 cc. of 0.1 N colloidal MnO_2 , and the required amount of H_3PO_4 or H_2SO_4 were mixed in a flask and the volume was brought up to 100 cc. The E_{H} values for 0.067, 0.167, and 0.333 M H_3PO_4 solutions were 1.144, 1.176, 1.196 volts, respectively. In H_2SO_4 solutions of 0.2, 0.5, and 1.0 normality, the E_{H} values were 1.271, 1.306, and 1.321 volts, respectively.

These values lie approximately on a straight line if E_{H} is plotted against the logarithm of the hydrogen ion concentration or the pH, of the solutions. The yield of acetaldehyde, however, does not vary so regularly, although the tendency is in that direction. It is probable that the change in hydrogen ion concentration, in addition to its effect upon the oxidizing agent, also affects the substance reduced (8), in this case, lactic acid.

Effect of Interfering Substances.

The effect of interfering substances, in general, is about the same as by the older procedure. Those compounds which by the older procedure yield none or less than 1 per cent of bisulfite-binding substances yield about the same amount by the newer

TABLE II.

Oxidation of Peptone and Peptone-Meat Extract Culture Media.

5 cc. of culture medium; 10 cc. of 10 per cent MnSO_4 ; 4 cc. of 1.67 M H_2PO_4 ; H_2O to 100 cc. Oxidation by 0.1 N MnO_2 . The results are expressed as mg. of lactic acid per 100 cc. of culture medium.

Method used	Aeration.				Distillation.	
Previous treatment of solution.....	None.		PWo* filtrate.		None.	
Acidity of lactic acid solution.	$\text{N H}_2\text{SO}_4$.	0.067 $\text{M H}_2\text{PO}_4$.	$\text{N H}_2\text{SO}_4$.	0.067 $\text{M H}_2\text{PO}_4$.	$\text{N H}_2\text{SO}_4$.	0.067 $\text{M H}_2\text{PO}_4$.
	(1)	(2)	(3)	(4)	(5)	(6)
Peptone-meat extract culture medium (C).....	83.8	55.3				
Ether extraction.						
I. Ether extract.....	46.2	48.2				
II. Residue.....	34.5	16.0	24.4	16.7	46.7	18.3
1.0 per cent peptone culture medium (A).....	28.2	5.8	20.1	2.0		
Ether extraction.						
I. Ether extract.....		1.4				
II. Residue.....	29.8	3.6	26.5	2.3		

* The phosphotungstic acid filtrate was made up of 20 cc. of culture medium, 5 cc. of 10 per cent phosphotungstic acid, 20 cc. of 5 N H_2SO_4 , and enough water to bring the volume to 100 cc. The acidity due to H_2SO_4 was neutralized by NaOH before adding the H_2PO_4 .

procedure.⁷ The sugars, saccharinic acids, and other sugar derivatives give approximately the same result.

A great difference, however, is noted in the oxidation of certain nitrogenous substances. This is shown by directly oxidizing such

⁷ This is true only when a well cooled condenser is used.

complex mixtures as bacterial culture media and urine. The results of oxidation of untreated and ether-extracted culture media are shown in Table II. These culture media contain 1000 mg., or more, of peptone and other nitrogenous compounds per 100 cc. Striking differences are noted if separate oxidations are carried out in $N H_2SO_4$ and 0.067 $M H_3PO_4$. The yield of bisulfite-binding substances is high when the oxidation is carried out in $N H_2SO_4$. This can be greatly diminished by precipitation with phosphotungstic acid. The greatest reduction, however, is noted when the oxidation occurs in H_3PO_4 . Although phosphotungstic acid has a favorable effect on the oxidation in $N H_2SO_4$ practically no difference is observed in H_3PO_4 . The favorable effect of low acidity is further shown by the fact that only slightly more of bisulfite-binding substance is obtained by distillation, whereas in $N H_2SO_4$ the result is considerably increased.

Determination of Lactic Acid in Various Materials.

A study of the methods heretofore used for the determination of lactic acid shows that the oxidation has been carried out in strongly acid solution. The methods of Boas, von Fürth and Charnas, Clausen, Friedemann, Cotonio, and Shaffer, and others call for an oxidation in a solution of H_2SO_4 of at least 1 per cent strength. The effect of acid concentration, although perhaps recognized, does not appear to have been studied. From the results presented it is evident that the oxidation may be carried out just as well at a considerably lower acid concentration. As a matter of fact, *the yield at the lower acid concentration is higher, less subject to variation, and the yield of other bisulfite-binding substances from nitrogenous compounds is greatly diminished.* The end-points also are sharper and more permanent.

Still another advantage is gained by carrying out the oxidation at the lower acid concentration. The recovery of added lactic acid from a 1 per cent peptone solution approaches that from a pure solution of lactic acid. However, if the oxidation is carried out in $N H_2SO_4$, the recovery may be less than 90 per cent. Such effects are not noted with non-nitrogenous materials. It appears to be due to the simultaneous oxidation of the non-protein nitrogenous materials in the presence of the strong acid.

The results of analyses illustrating these points are shown in

Table III. Peptone-meat extract, a culture medium commonly used in bacteriological work, was oxidized directly without any preliminary treatment both before and after adding lithium lactate. The details and conditions are shown in Table III. The oxidation in $N H_2SO_4$ resulted in a recovery of only 87 per cent as compared with about 95 per cent when the oxidation was carried out in H_3PO_4 solution. The favorable effect of removing some of

TABLE III.

Recovery of Added Lactic Acid from 1 Per Cent Peptone-0.3 Per Cent Meat Extract Culture Medium.

5 cc. of culture medium; 10 cc. of 10 per cent $MnSO_4$; 4 cc. of 1.67 $M H_3PO_4$; H_2O to 100 cc. Oxidation by 0.1 $N MnO_2$. The results are expressed as mg. of lactic acid per 100 cc. of culture medium.

	Direct, no treatment.		PWo* filtrate.	
	$N H_2SO_4$.	0.07 $M H_3PO_4$.	$N H_2SO_4$.	0.067 $M H_3PO_4$.
Culture medium (M).....	73.0	48.2	59.3	45.0
“ “ plus 180 mg. lactic acid....	228.9	218.0	220.9	216.2
Lactic acid recovered.....	155.9	169.8	161.6	171.2
Per cent recovery.....	86.6	94.3	89.8	95.1
Culture medium (N).....	68.8	48.6		
“ “ plus 90 mg. lactic acid....	147.0	135.3		
Lactic acid recovered.....	78.2	86.7		
Per cent recovery.....	86.9	96.3		
Lactic acid standard, per cent recovery.....	93.0	98.1		

* Phosphotungstic acid filtrate. See Table II.

the nitrogenous substances is shown by the analysis after precipitation by phosphotungstic acid. The recovery increased by 3 per cent in $N H_2SO_4$ and a smaller increase of about 1 per cent was also noted in the oxidation carried out in H_3PO_4 . The loss in recovery in the two series of analyses shown, compared with the oxidations of pure lactic acid under identical conditions, is about 6 per cent in $N H_2SO_4$ and about 3 per cent in H_3PO_4 .

While the results of oxidations in H_3PO_4 shown in Table III are

not entirely satisfactory, it must be remembered that they represent an extreme. The materials most often studied are blood and muscle, and the filtrates which are finally analyzed contain relatively small amounts of nitrogenous substances. The effect of their presence on the yield is therefore quite small. Nevertheless it is apparent that even in the extreme case (peptone-meat extract)

TABLE IV.
Analysis of Urine.

10 cc. of urine (or an equivalent amount of filtrate or extract); 10 cc. of 10 per cent MnSO_4 ; 2 cc. of 2 M H_3PO_4 ; H_2O to 100 cc. Oxidation by 0.1 N MnO_2 .

	Mg. per cent of lactic acid.	End-point.
Direct oxidation of untreated urine...	26	Fades very rapidly.
Phosphotungstic acid filtrate.....	26	" " "
$\text{CuSO}_4\text{-Ca(OH)}_2$ filtrate.....	9.7	End-points sharp and permanent.
Ether extract.		
Direct oxidation.....	8.1	" "
$\text{CuSO}_4\text{-Ca(OH)}_2$ filtrate.....	5.5	" "

TABLE V.
Analysis of Urine.

Analysis of the $\text{CuSO}_4\text{-Ca(OH)}_2$ filtrate. Oxidation by 0.1 N MnO_2 . The results are expressed as mg. of lactic acid per 100 cc.

Method.	N H_2SO_4 .	0.13 M H_3PO_4 .
Aeration.....	24	13.3
Distillation.....	40	12.7

the results are more reliable when the oxidation is carried out at the lower acidity.

The analysis of urine presents a number of interesting points. The substances present in largest amount, urea, creatinine, and uric acid, yield no bisulfite-binding substances on oxidation (3). This is true also of many other substances which are present in smaller concentration (3). Nevertheless the direct oxidation of urine in the presence of N H_2SO_4 results in the formation of a very

large amount of bisulfite-binding substances, but they are of such a nature as to indicate that the products have a very small affinity for the bisulfite. The first end-point fades very rapidly, and becomes fairly permanent only after long continued addition of iodine. A smaller amount of such substances is obtained if the oxidation is carried out in H_3PO_4 . Phosphotungstic acid, although it removes a large part of the nitrogenous materials present, apparently does not remove the materials which give rise to these bisulfite-binding substances. They are, however, largely removed by $\text{CuSO}_4\text{-Ca(OH)}_2$, and such a filtrate gives values for lactic acid

TABLE VI.
Analysis of Blood.

The oxidations were carried out either with 0.0025 N KMnO_4 or 0.01 N MnO_2 as indicated in the table. The results are expressed as mg. per cent of lactic acid.

Animal.	Tungstic acid filtrate.		$\text{CuSO}_4\text{-Ca(OH)}_2$ filtrate.			
	0.1 M $\text{H}_3\text{PO}_4 + \text{MnO}_2$.		0.1 M $\text{H}_3\text{PO}_4 + \text{MnO}_2$.		N $\text{H}_2\text{SO}_4 + \text{KMnO}_4$.	
	Aeration.	Distillation.	Aeration.	Distillation.	Aeration.	Distillation.
Sheep.....	23	26	21	27	27	31
Dog*.....	12	16	10	10	12	14
Man†.....			8	7	9	10

* Blood taken 5 days after double nephrectomy. The animal was very quiet as in coma. Non-protein nitrogen 273 mg. per cent.

† Sample taken after 45 minutes of sleep.

which agree fairly well with the values obtained on the ether extract. Both titration end-points in the latter instances are sharp and permanent (see Table IV).

The advantage of carrying out the oxidation at the lower acidity is again illustrated in Table V. The $\text{CuSO}_4\text{-Ca(OH)}_2$ filtrate from urine was analyzed by two procedures, with the apparatus and aeration recommended by Friedemann, Cotonio, and Shaffer and by simple distillation. The details are described in the table. With the aeration method, the results were almost twice as high from N H_2SO_4 as from H_3PO_4 . They were even higher when the distillation method and H_2SO_4 were used. The two methods gave identical results in H_3PO_4 .

The effect of the acid concentration on the oxidation of blood filtrates is not so striking as in the two extreme cases just cited. Nevertheless small differences are noted, especially when the lactic acid concentration is low, as in the three series of analyses shown in Table VI. Analyses were made on both the tungstic acid and $\text{CuSO}_4\text{-Ca(OH)}_2$ filtrates. The various procedures and the conditions used are shown in the table. The lowest values were obtained by oxidation in H_3PO_4 , by the aeration method of Friedemann, Cotonio, and Shaffer. Triplicate analyses made by this procedure agreed more closely, and the end-points were sharper and more permanent, than by any of the other procedures used.

SUMMARY.

1. A procedure for the determination of lactic acid is described.
2. The factors which influence the precision of the method are discussed.
3. The effect of certain types of interfering substances upon the yield of lactic acid is demonstrated.
4. Data illustrative of the amount of lactic acid in various biological preparations are included.

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THE DETERMINATION OF CARBON AND CARBON DIOXIDE.

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One of the desirable additions to the armamentarium of chemistry is a rapid, simple, and precise method for the determination of carbon. At the present time there seem to be but two outstanding methods for this purpose, the dry combustion method of Liebig and the so called Messinger (1-3) method, the latter permitting of combustion in the wet way.

The dry combustion method does not lend itself well to organic substances in solution, and it is frequently difficult or impossible to remove liquids by feasible procedures, leaving all of the carbon behind in the residue in suitable condition for analysis. This applies particularly to some of the materials of biochemical importance, as for example, urine, blood, tissues, and bacterial culture media. Desiccation of the materials enumerated invariably is accompanied by loss of CO_2 and some of the other more volatile substances, such as alcohol and the lower fatty acids. For such complex mixtures the wet combustion method would seem to be preferable, since the oxidation may be carried out without the preliminary removal of water. It is found, however, that water which is present in the material analyzed interferes in the oxidation of many substances by the chromic-sulfuric acid mixture. This is particularly true of such substances as acetic acid and the fats. To obviate this difficulty, the oxidation with chromic-sulfuric acid in the presence of silver (4) or mercury (5) salts to act as catalysts, has been suggested.

It is significant that the majority of favorable results recorded in the literature (3, 6-8) has been obtained when the acid concentration is relatively high, and the volume of water small, or

even absent. On the other hand, it is equally significant that at least a majority of unsuccessful attempts to use the method has been those in which the amount of acid is relatively small, and the volume of water relatively large (9, 10). These discrepancies, due presumably to the water present, have suggested the work here presented. A study has been made of the effect of water and of acid upon the precision of the wet combustion method. The results obtained show quite convincingly that both acid concentration and volume of water affect the precision of the procedure. When proper compensation is made for the water present the carbon content of even such difficultly oxidizable substances as acetic acid and fats may be determined with satisfactory results.

The apparatus described consists of a reaction flask, a reflux condenser, and an absorbing system for CO_2 . It is essentially the apparatus described by Messinger (3), in 1890, but simplified, and in some of its details is like that of Ames and Gaither (10). The oxidizing reagents consist of chromic, sulfuric, and phosphoric acids, used in the proportions recommended by Schollenberger (11). Carbon dioxide resulting from the oxidation, is absorbed by 0.5 N NaOH in a tower of special construction and determined by titration with 0.5 N HCl after addition of an excess of BaCl_2 (12).

Description of Method.

Apparatus.

The construction of the apparatus is shown by Fig. 1. A single unit is shown; it is convenient and economical to have several units mounted on a common frame.

A is a 300 cc. Kjeldahl flask fitted with a rubber stopper through which pass Tube B, and C, a Hopkins condenser. Tube B fulfils two purposes: the separatory funnel at the top permits of the addition of the oxidizing mixture; the side arm is connected by rubber tubing with the carbon dioxide-free air main. The Hopkins condenser connects by rubber and glass tubing with the flask, D, of 500 cc. capacity. Emerging from flask D is the absorption tower, E, containing glass beads. The latter are held in place by a perforated porcelain plate, and are solid and of two sizes, respectively, 2 and 3 mm. in diameter. The absorption tower is connected in turn with the tube, F, partly filled with water. This is a safety tube, also indicating the

rate of flow of the carbon dioxide-free air through the apparatus. This is regulated by clamp *G*, on the rubber tubing which joins

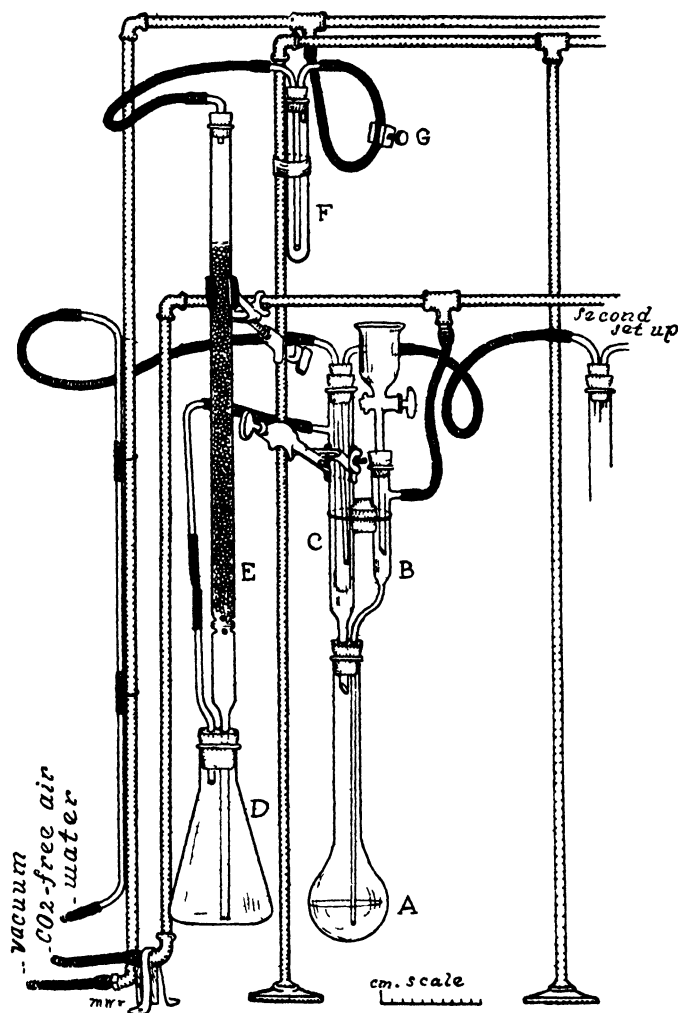


FIG. 1. Apparatus for determination of total carbon and carbon dioxide.

the tube, *F*, to the vacuum pump. The vacuum pump draws a current of carbon dioxide-free air from the main through the side tube in *B* to flask *A*. From here it passes, together with

carbon dioxide liberated during the digestion, to the Hopkins condenser, where water vapor is condensed, and falls back into flask *A*. The carbon dioxide and air pass to flask *D*, and upwards through the absorption tower, which contains a definite amount of standardized caustic soda. During its passage through the tower, the carbon dioxide is absorbed by the alkali, and the residual air passes away through tube *F* to the vacuum pump.

The entire apparatus is suspended by means of two burette clamps. These permit of sidewise movement, a point of importance in attaching flasks *A* and *D*. The frame for the apparatus is made of $\frac{1}{4}$ inch iron pipe, joined by elbows and T's. It is attached to the desk by flanges.

Carbon dioxide-free air is supplied through two 5 foot towers made of 2 inch iron pipe. The ends are capped, and tapped to fit $\frac{1}{4}$ inch nipples. These make convenient attachments for pressure tubing connecting with the air mains of the apparatus. These towers are filled with moistened soda-lime, about equal portions of 4 and 8 mesh.

Micro burners, connected to the gas mains through $\frac{1}{8}$ inch valves, provide satisfactory and adjustable sources of heat.

Reagents.

1. *Chromic Acid*.—340 gm. of chromic acid (CrO_3) are dissolved in 400 cc. of hot CO_2 -free distilled water and made up to a volume of 1 liter with 85 per cent phosphoric acid.

2. *Sulfuric-Phosphoric Acid*.—Equal volumes of concentrated c.p. sulfuric acid and 85 per cent phosphoric acid are mixed, cooled, and kept in a glass-stoppered bottle.

3. *Standard NaOH Solution*.—An approximately 0.5 N solution.

4. *Standard HCl Solution*.—An exactly 0.5 N solution.

5. *Phenolphthalein*.—A 1 per cent alcoholic solution.

6. *Molar Barium Chloride Solution*.

7. *CO_2 -Free Distilled Water*.—Distilled water is boiled 10 to 15 minutes and allowed to cool.

Procedure.

A sample containing from 25 to 100 mg. of carbon is weighed or measured in the 300 cc. Kjeldahl flask and the flask is connected to the apparatus. Next, 75 cc. of standard alkali are measured into a 500 cc. Erlenmeyer flask and connected to the tower. The

aeration is started and regulated by clamp *G* so that air passes through tube *F* at the rate of 50 to 150 bubbles per minute. 10 cc. of chromic acid solution are allowed to run into the flask through the separatory funnel. This is followed by 50 cc. of sulfuric-phosphoric acid mixture. In case there is water in the sample, 2 cc. of concentrated H_2SO_4 for every cc. of water present are also added. The contents of the flask are mixed by gentle rotation. Heat is applied, very gently at first, and then gradually increased until the solution boils. This usually requires about 20 minutes. If the reaction becomes too violent, as is indicated by the tendency of the reaction mixture to rise in the inlet tube, the flame is removed and heating is resumed only when air again bubbles through the solution. Boiling is continued until oxidation is complete. In the case of difficultly oxidizable substances, such as fats, it may be necessary to add more chromic acid after the solution has boiled for some time.¹ In the case of easily oxidizable substances, such as the sugars, a total digestion period of 30 minutes suffices. Proteins require a somewhat longer digestion period, and it is advisable in the case of substances such as fats to continue the boiling for 1 hour.

The flame is removed, the suction is discontinued, and the reaction flask is disconnected. The stopper at the top of the absorption tower is next removed, and about 200 cc. of carbon dioxide-free water, in 25 to 50 cc. portions, are run in. The flask is removed from the tower, about 25 cc. of *M* BaCl_2 are added, and the flask is shaken and stoppered. The precipitate of BaCO_3 becomes granular after a few minutes standing. The residual alkali is then slowly titrated with 0.5 *N* HCl , with phenolphthalein as an indicator, to the *total disappearance of the pink color*. The flask must be shaken or rotated constantly while the acid is being added.

Frequent blanks should be run. The blank corrects for CO_2 in the apparatus and reagents, and CO_2 absorbed from the atmosphere during the titration.² It is unnecessary to use accu-

¹ Chromic acid decomposes when heated with strong sulfuric acid.

² The blank also corrects for the small amount of acid carried over in white fumes. According to Küster and Stallberg (7) the white fumes may be removed by a tube filled with glass wool. More efficient, however, is a tube filled with small glass beads which may be inserted between the reflux condenser and the absorbing tower.

rately standardized NaOH; the same volume, however (measured from an automatic burette) should be present in all determinations, including the blank. The titrations may be expressed in terms of the volume of 0.5 N HCl used. The calculation then is made as follows: Volume of HCl used in blank titration — volume of HCl used in titration of sample = HCl equivalent to alkali combined with CO_2 . 1 cc. of 0.5 N HCl (which must be accurately standardized) is equivalent to 3 mg. of carbon.

Determination of Carbon Dioxide.

It is obvious that the apparatus lends itself equally as well for the determination of carbon dioxide as for total carbon. The

TABLE I.
Determination of Carbon Dioxide.

50 cc. of 0.5 N NaOH and 25 cc. of water were used in the tower in all determinations.

Weight of Na_2CO_3 .	Volume of HCl* equivalent to NaOH used up.	Calculated volume of NaOH used up.	Recovery.
gm.	cc.	cc.	per cent
0.2530	9.52	9.51	100.1
0.2530	9.49	9.51	99.8†
0.2530	9.52	9.51	100.0
0.5059	19.02	19.01	100.0
0.5059	19.00	19.01	100.0

* 1 cc. is equivalent to 0.0266 gm. of Na_2CO_3 .

† The rate of titration with HCl in this case was very rapid.

results shown in Table I were obtained by decomposing pure Na_2CO_3 with HCl. The CO_2 liberated was aspirated through the towers which contained 50 cc. of 0.5 N NaOH and 25 cc. of water. The recovery of CO_2 was found to be quantitative only when certain precautions were observed.

The rate of aspiration apparently does not affect the absorption. Complete absorption has been obtained even with a rate of 300 to 400 bubbles of air passing through tube *F*. The rate of titration, however, may have an influence on the results. If run in very rapidly, the local concentration of HCl may become so great as to decompose some of the BaCO_3 . In consequence of this, more HCl is used and the calculations show a lowered yield.

Most important, however, is the construction of the tower and the concentration of alkali. Carbon dioxide is rapidly absorbed only by concentrated solutions of alkali. The absorption is very

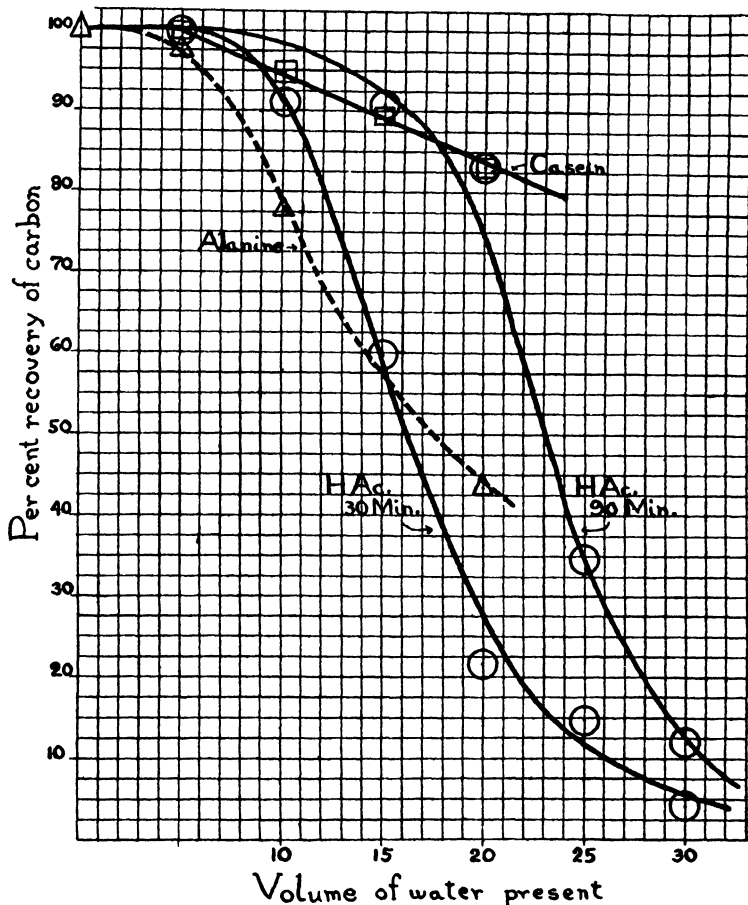


CHART I. Effect of water on the oxidation of acetic acid, alanine, and casein.

markedly influenced by the concentration, and in dilute alkali the absorption is slow and incomplete. A quantitative absorption can be obtained if both the time of contact with liquid and the surface are increased, as can be done, for example, by

aspirating the gas through a tower containing beads. The height of the column of beads in the tower is important. But more important still is the excess of alkali which must be maintained. With the tower described and the 0.5 N NaOH, an excess

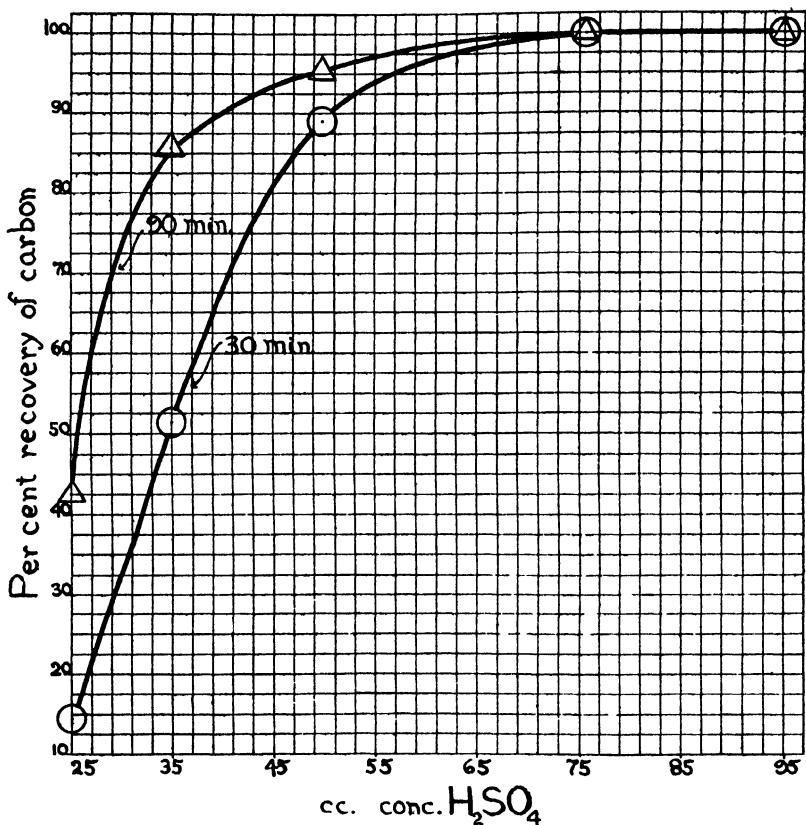


CHART II. Relation between volume of water, volume of H_2SO_4 , and yield of CO_2 from the oxidation of acetic acid.

of at least 25 cc. of alkali over that necessary for complete absorption should be present.

Effect of Water and Acid upon Oxidation.

The effect of water added to the oxidizing reagents is shown in Chart I. Solutions of alanine and acetic acid were measured

into 300 cc. Kjeldahl flasks, and enough additional water was added to bring the volume to the quantity indicated in the chart. Casein was weighed out, transferred to the flask, and the indicated volume of water was added. The oxidation was carried out with 10 cc. of chromic acid solution and 50 cc. of sulfuric-phosphoric acid mixture. Except in the case of acetic acid, the total

TABLE II.
Recovery of Carbon as Carbon Dioxide by Oxidation of Pure Organic Compounds.

99 to 100 per cent.	98 to 99 per cent
Acetic acid. Ethyl alcohol. Crotonic acid. β -Hydroxybutyric acid. Lactic acid (lithium salt). Potassium hydrogen saccharate. Sugars. Sucrose. Glucose. Galactose. Rhamnose. Methyl amine HCl. Alanine. Aspartic acid. Uric acid. Urea. Dimethylglyoxime. Chinchonine. Potassium hydrogen phthallate. Benzoic acid. Hydroquinone. Piperine.	Stearic acid. Carbazole.

time of digestion was 30 minutes. It is apparent that water very markedly affects the yield, presumably by decreasing the rate of oxidation.

The unfavorable effect of water may be overcome by adding concentrated H_2SO_4 to the oxidizing mixture. This is illustrated by the results of oxidation of acetic acid shown in Chart II. 5 cc. of a solution of acetic acid and 20 cc. of water were measured into

Kjeldahl flasks, and the usual amounts of oxidizing reagents, namely 10 cc. of chromic acid solution and 50 cc. of sulfuric-phosphoric acid mixture, were added. 10, 25, 50, and 75 cc. of additional concentrated H_2SO_4 were added. The total volume of H_2SO_4 present in the flask therefore was 25 cc. plus the amount of additional acid. The results of 30 and 90 minutes digestion on the yield are shown in Chart II. As can be seen, the unfavorable effect of water can be compensated for by increasing the concentration of sulfuric acid. 50 cc. of additional H_2SO_4 in this case were sufficient to bring about complete oxidation even when 25 cc. of water were present.

Oxidation of Pure Organic Substances.

The degree of accuracy of the procedure is illustrated by the analyses shown in Table II. Except for piperine, hydroquinone, and chinchonine, the recovery of carbon from the substances listed in the first column was quantitative or almost quantitative. The yield from stearic acid and carbazole was somewhat low. Both of the substances are very insoluble. In the case of stearic acid it was noted that a very small amount, which could not be dislodged, was always in the condenser at the end of the oxidation. Carbazole showed a tendency to creep upward and stick to the side of the flask. In spite of every attempt to wash it down by rotation of the flask during the digestion, a small amount always remained in the upper part of the flask.

SUMMARY AND CONCLUSIONS.

An apparatus and a procedure for the determination of total carbon and carbon dioxide is described. It is pointed out that the wet combustion method is better adapted for materials of biochemical importance than the dry combustion method. The results are quite quantitative, and such difficultly oxidizable substances as acetic acid and fats may be completely oxidized without using catalysts, such as silver or mercury, provided proper attention is paid to the total acid concentration.

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STUDIES ON CHOLESTEROL.

IV. THE RELATION OF OVARIES AND TESTES TO CHOLESTEROL METABOLISM.

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In a previous paper (1) we reported that removal of neither the spleen nor the suprarenal glands from white rats had any effect upon the cholesterol content of the blood. In a further attempt to throw light on cholesterol metabolism and especially the site of cholesterol synthesis in the body we have studied the effect on the blood cholesterol of removing the testes from male and the ovaries from female rats.

In the literature very little is found on this subject. Parhon and Marza (2) found no difference in the cholesterol of the serum from normal and castrated sheep but in the cock the castrated animals showed a definite hypocholesterolemia. Parhon and Parhon (3) had previously reported a marked drop in the cholesterol content of the blood of a 75 year old man following a testicular implantation.

EXPERIMENTAL.

In this as in our previous experiments it seemed advisable to make use of our cholesterol-free diet (4). Adult rats of varying ages (4 months to 1 year) were used in the experiments and after removal of the testes or ovaries were kept on the cholesterol-free diet for periods of 1 week, 2 weeks, and 3 weeks respectively. Another group after operation were put on the same diet with 0.1 per cent cholesterol and kept on experiment for 2 weeks.

We also used young rats that were 1 month of age and after operation kept them on experiment for 2 months until they reached the age of sexual maturity. Our reason for using the

young rats is because of the fact that castration performed before the age of sexual maturity produces quite different results from those produced by the same operation after the age of sexual maturity is passed. Of the younger rats two of each sex were submitted to the operative procedure without removal of either the testes or ovaries. In this operation the same incision was made; the gonads were dissected out and manipulated but were not removed. As a further control two young rats of each sex were

TABLE I.
Effect of Removal of Testes and Ovaries on Blood Cholesterol.

No. of rats used.	Age.	Operation.	Diet.	Duration of experiment.	Cholesterol per 100 cc. of blood, average for group.
					mg.
4	Adult.	Testes removed.	Cholesterol-free.	1 wk.	89
4	"	" "	"	2 wks.	86
4	"	" "	"	3 "	87
4	"	" "	0.1 per cent cholesterol.	2 "	88
6	1 mo.	" "	Cholesterol-free.	2 mos.	93
4	Adult.	Ovaries "	"	1 wk.	88
4	"	" "	"	2 wks.	88
4	"	" "	"	3 "	96
4	"	" "	0.1 per cent cholesterol.	2 "	87
5	1 mo.	" "	Cholesterol-free.	2 mos.	86
4	1 "	Control, operative procedure.	"	2 "	91
4	1 "	Control, no operation.	"	2 "	86

kept for 2 months on the cholesterol-free diet without having been operated at all. For the adult rats no non-operated controls were used as we had already considerable data on normal adult animals.

At the end of the allotted time following operation the rats were killed and the blood was analyzed for cholesterol content. In most cases it was possible to obtain sufficient blood for separate determinations on each rat. (In the few cases where this was not

possible the blood of two rats of the same series was mixed and determinations made on the mixture. In all cases duplicate determinations were made and if the results did not check within reasonable limits the determinations were repeated.) The method of Bloor (5) was used for the cholesterol determinations with the exception that a composite sample of alcohol-ether blood extracts of known cholesterol content was used as the standard in all determinations. This modification was suggested in our previous paper (1). The results of the determinations are shown in Table I.

In previous work on the cholesterol content of rat blood we have found the average figure for a large series of normal rats to be 88 mg. per 100 cc. of blood. Comparing this with the figures in Table I shows that there is practically no change in the cholesterol content of rat blood following the removal of either the testes from males or the ovaries from females. Nor does it make any difference whether the removal occurs before or after the rat reaches the age of sexual maturity.

SUMMARY.

Experiments are described showing that removal of the testes from male or the ovaries from female rats has no appreciable effect on the cholesterol content of the blood.

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CHEMICAL STUDIES OF MUSCLE CONTRACTURE.

II. THE DISTRIBUTION OF PHOSPHORUS IN FROG MUSCLE DURING DELAYED RELAXATION.

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(Received for publication, February 1, 1929.)

Fatigue contracture is produced in an isolated frog gastrocnemius when it is stimulated electrically until it no longer responds to the stimulus. A kymographic record of the contracture shows that the apparent failure in response of the muscle is due to a failure in relaxation, for the height of the contracture is often higher than the initial contraction. Cessation of the stimulus is followed by a relatively slow relaxation.

A condition which simulates fatigue contracture can be produced in frog muscles by the intraperitoneal injection of hypertonic solutions of non-toxic substances. The requirement of the ideal contracture so produced is that the extirpated muscle shortens immediately when the first stimulus is applied and relaxes so slowly that subsequent stimuli applied at an appropriate rate reach it during the shortened state. In order that the criterion for the production of contracture be adequate, the rate of stimulation must be well below the tetanic rate for an excised muscle from an untreated frog. A rate of about thirty shocks per second is required to produce complete tetanus in a previously unstimulated frog gastrocnemius. In the present work a rate of six per second has been used and we believe this rate is sufficiently slow to indicate a marked change in relaxation time when it produces tetanus.

The earliest observations of the effect of water-absorbing substances on the musculature of frogs appear to have been made in 1866 by Hause-

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mann and Umethun (quoted by Santesson). These investigators found that the subcutaneous injection of large doses of glycerol, and of concentrated solutions of sodium chloride or sugar caused muscular rigidity to develop. Santesson (16) has given a review of the literature on the effects of glycerol and of veratrin on frog muscle, and found in his own work that glycerol had a direct action on the muscle which predisposed it to the development of contracture, and agreed with earlier workers that this action was associated with dehydration.

In 1884 Ringer (14) found that intraperitoneal administration of sodium phosphates and of sodium bicarbonate in frogs caused dystonic movements of the limbs and rigidity. When the gastrocnemii were isolated from the animals and stimulated directly by an electric current they showed an increased relaxation time. It appears, however, that he did not connect the phenomenon with dehydration but rather with a specific effect of the bicarbonate and phosphate ions.

The observation of Urano (19) made in 1907 regarding the relation of phosphoric acid and creatine in living muscle is, in light of recent discoveries, prophetic. To quote, "Die oben angedeutete Vermutung dass Kreatin und Phosphorsäure aus demselben im lebenden Muskel vorgebildeten Komplex stammen, ist sonach nicht schlechterdings zurückzuweisen." His conclusions were based on the rate of diffusion of the two substances out of living and dead muscle.

Throughout the literature on muscle tonus the idea has been expressed that creatine is in some way connected in physiologic function to muscle tone. Pekelharing (13) found an increased excretion of creatine in the urine of men following prolonged standing in the military position of attention, and it appeared logical to conclude that the creatine was set free by the tonic muscles and then excreted. Mitsuda and Uyeno (11) reported an increase in creatine in frog muscles during nicotine contracture. Scaffidi (17), however, found that fatigue did not alter the creatine content of muscle.

In view of recent work it seems most probable that in resting muscle practically all of the creatine exists in combination with phosphoric acid, and if we recall Urano's findings, the slow diffusibility of the two substances from living muscle indicates that phosphocreatine is bound by protein. The creatine content of rabbit muscle was found to be 367 mg. per cent by Cabella (2) and 528 mg. by Baumann and Hines (1). An approximate average of these values shows the creatine concentration in muscle to be 0.03 M. The maximum phosphocreatine concentration found by Davenport and Sacks (4) in the same sort of muscle was 0.027 M, a value which agrees within the limitations of technical error with the concentration of creatine itself. Hence the relationship between bound and free creatine should have greater significance in the physiology of muscle than total creatine.

In the present study the reckoning of phosphocreatine may be taken as a guide to the behavior of creatine, as well as phosphoric

acid, and we shall endeavor to show that a low phosphocreatine value is related to the tendency of frog muscle to show contracture.

EXPERIMENTAL.

Individuals of *Rana pipiens* which weighed about 100 gm. were used for all experiments. They were caught during the months of October and November and were used for the experiments within 1 to 3 weeks after capture. Except when otherwise stated, they were kept in an aquarium at room temperature (25°) but were supplied with running water at a temperature of about 10°.

The physiologic data were obtained in the form of kymographic tracings when the muscle was loaded relatively isotonicly with 50 gm. by the following method. The inertia of the load was reduced to an insignificant value by suspending a weight of 50 gm. to the writing lever by means of a rubber band 1.5 mm. thick. When the muscle had been clamped to the rigid frame of the apparatus with a bone clamp attached to the femur, the 30 gauge wire leading to the writing lever was secured to the calcaneus tendon by a small hook, the lever was adjusted for height, and the 50 gm. weight then clamped at whatever height it happened to be when hanging freely on the rubber band. Such a system was not strictly isotonic since the muscle reacted against a 50 gm. tension on the band.

Direct electrical stimulation from the secondary of an induction coil was transmitted through two silver electrodes at each end of the muscle. The strength of the stimulus was adjusted to be just maximal for the average normal muscle. The primary circuit interrupter was adjusted to give makes and breaks uniformly spaced at the rate of 3 each per second. The temperature of the muscle during stimulation was approximately 25° so that a low temperature (which is known to favor contracture) was not a factor.

All muscles were prepared for chemical analyses by first freezing with a slush of carbon dioxide snow and ethyl chloride, then cross-sectioning by hand with a thin bladed laboratory knife into slices preferably not thicker than 0.2 mm., and dropping the still frozen sections into 5 per cent trichloroacetic acid solution. A complete description of the technique has been reported previously (3, 15). On account of the small size of the muscles, it was

necessary to limit the number of determinations of phosphorus to the inorganic, phosphocreatine, and total acid-soluble values. Inorganic phosphate was determined by the procedure of Sacks and Davenport (15), the A values by direct readings on the filtrates, and the total phosphorus by wet ashing aliquots of the filtrate with 0.5 cc. of concentrated sulfuric acid, eliminating the carbonaceous material with a few drops of 2 per cent phosphorus-free hydrogen peroxide, and adding (after diluting) molybdate solution and reducing agent to the digested material. The colorimetric method of Fiske and Subbarow (7) was used throughout.

Direct comparisons of data have been made between muscles obtained from the same animal. The conditions of comparison were somewhat variable and hence require a rather detailed description. We endeavored to establish an average value for the inorganic, phosphocreatine, and total phosphorus in resting, untreated frogs of the type to be used. The spinal cord was blocked by injecting about 0.1 cc. of 95 per cent ethyl alcohol into it in the lumbar region. The gastrocnemius of one leg was frozen while the skin was left intact, the limb ligated near the pelvic articulation, and amputated. After 2 hours the second gastrocnemius was frozen and removed in the same manner. This procedure established a control type of experiment for subsequent work in which contracture-producing substances were administered to the frogs after the removal of the first limb. It was found that the amputation of the first limb did not in itself affect the distribution of phosphorus compounds in the second limb during the time interval used. The inorganic phosphate found in the resting untreated muscles varied between 21 and 32 mg. per cent of phosphorus. The A value (phosphocreatine plus inorganic phosphate) varied between 71 mg. and 95 mg. and the totals between 138 mg. and 160 mg. Hence a phosphocreatine phosphorus value of about 60 mg. could be considered usual, and the remaining phosphorus fractions amounted to 65 mg. Muscles isolated from decapitate animals gave a slightly higher inorganic value, a lower A value (therefore less phosphocreatine) and a larger (T-A) remainder. The values for inorganic and phosphocreatine phosphorus are in very good agreement with the data obtained by Eggleton and Eggleton (6).

When an extirpated gastrocnemius was stimulated until it de-

veloped fatigue contracture, Fig. 1, 1S, and frozen during stimulation, the distribution of phosphorus in the fractions studied was

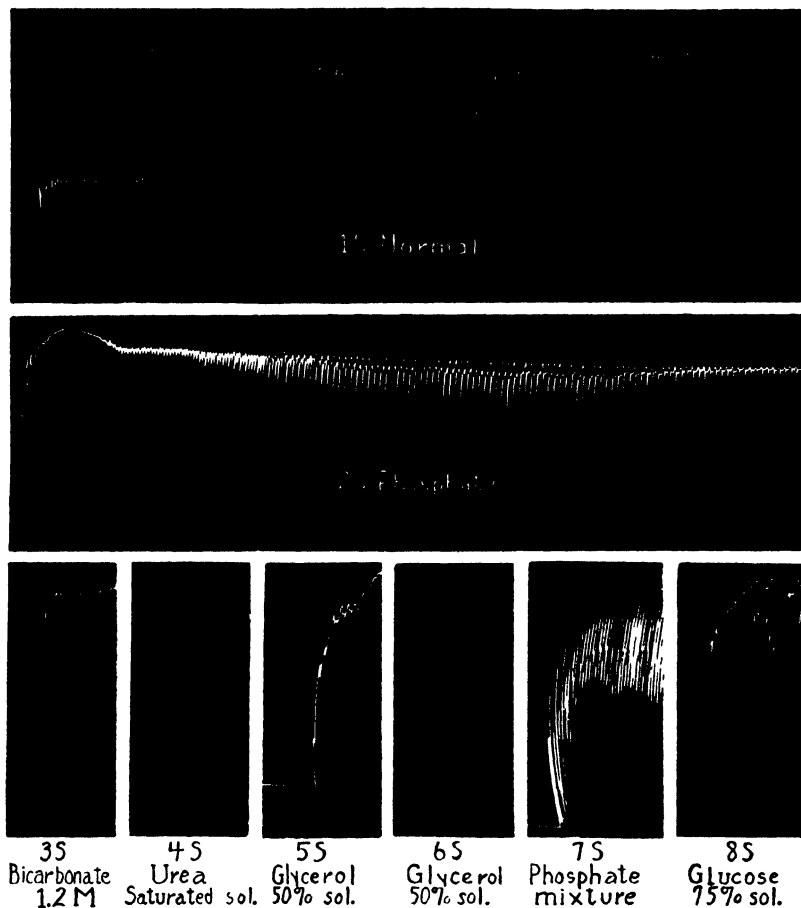
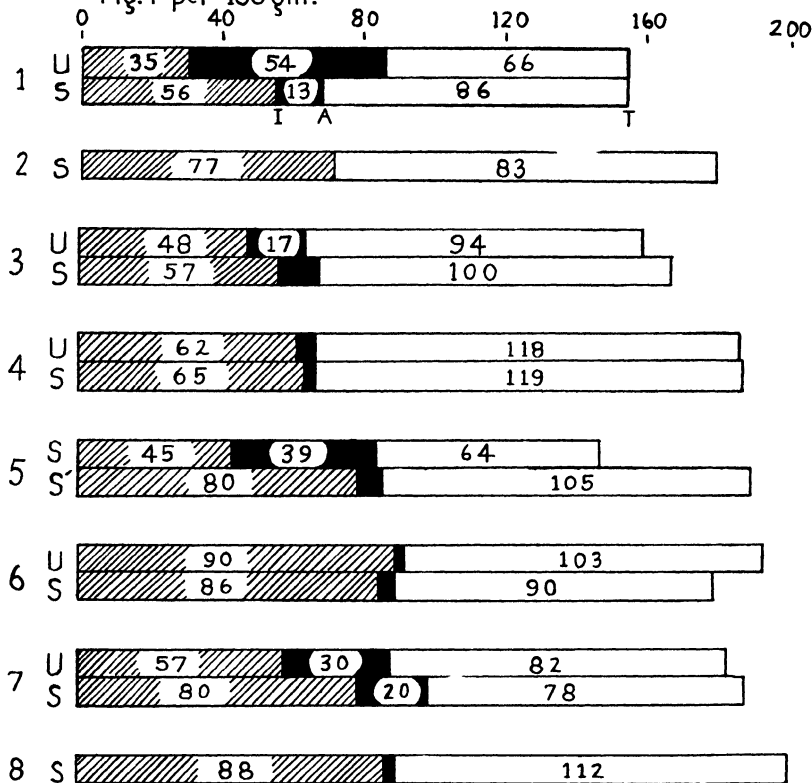


FIG. 1. Kymographic tracings of extirpated frog gastrocnemii. The lower line shows stimulation rate and time; the down stroke occurs at the make and the up stroke at the break in the primary. Each stroke equals $\frac{1}{2}$ second. The chemical data obtained at the time of freezing (end of each chart) are recorded with corresponding numbers in Fig. 2.

found to be as represented in Fig. 2, 1S. By comparison with the unstimulated gastrocnemius (1U) extirpated from the opposite

limb of the same frog one sees that the phosphorus of inorganic phosphate has risen from 35 mg. to 56 mg. The phosphocreatine



Legend

▨ Inorganic

□ Remaining acid-soluble

■ Phosphocreatine

FIG. 2. Graphic representation of the distribution of phosphorus in extirpated frog gastrocnemii. U, unstimulated; S, stimulated. 1U, extirpated, resting; 1S fatigue contracture from an untreated animal. 5S, brief stimulation before glycerol, 5S', similar stimulation after glycerol. All others unstimulated and stimulated from animals given hypertonic solutions listed in Fig. 1.

phosphorus has been reduced from 54 mg. to 13 mg. while there are 20 mg. which have been transferred to the more stable re-

mainder. This relatively large amount of phosphocreatine which was present in that particular muscle was higher than that usually found in fatigue contracture and probably includes some which was resynthesized anaerobically (10, 12) during a delay of a few seconds in completing freezing. A more extreme condition is seen in 2S (Figs. 1 and 2) wherein the phosphocreatine is absent following stimulation superimposed upon an artificially produced contracture. Here the determinations of the inorganic phosphate and the A value checked. Note that the latter is lower than for resting muscle even though there has been dehydration as indicated by the abnormally high total phosphorus.

By the procedure of simultaneously recording physiologic behavior with chemical findings we hoped to obtain information regarding the constancy with which the distribution of phosphorus compounds seen in fatigue contracture occurred in the contractures produced by dehydration.

A repetition of Ringer's (14) experiments gave variable results. The intraperitoneal injection of 5 cc. of a solution of a mixture of Na_2HPO_4 , 0.3 M, and NaH_2PO_4 , 0.05 M, caused twitching and some rigidity, but when the gastrocnemii were removed from frogs so treated and tested by stimulation at the rate of 6 shocks per second only relative degrees of contracture were obtained. Fig. 1, 7S, shows the record usually obtained while 2S shows the more extreme condition. The administration of 2 cc. of 1.2 M NaHCO_3 produced variable degrees of contracture and since it was more toxic than phosphate the animals frequently died within an hour. When the survivors were kept in a refrigerator overnight after bicarbonate administration and then warmed to room temperature, the extirpated muscles usually went into contracture on stimulation as shown in Fig. 1, 3S.

The degree of hydration of the muscles was checked by the determination of protein nitrogen in the tissue residues after extraction of acid-soluble phosphorus. The increase in nitrogen paralleled the increase in total acid-soluble phosphorus and varied from 10 to 30 per cent. In one frog the total phosphorus in the muscle removed before the administration of bicarbonate was 150 mg. per cent. 2 hours later the second leg was removed and a total phosphorus of 205 mg. found. This was the most extreme example of dehydration observed.

A review of our preliminary data suggested that the degree of contracture was related to the degree of dehydration, and that coincidentally there was usually a reduction in phosphocreatine in the resting companions of the muscles which developed definite contractures on stimulation.

Glycerol administered in doses of 2 cc. of 50 per cent solution caused marked muscular rigidity, and gastrocnemii removed in about 1 hour from frogs so treated responded to stimulation uniformly by contracture. Characteristic responses are shown in Fig. 1, 5S and 6S. Fig. 2, 5S shows the distribution of phosphorus compounds in one gastrocnemius before administration of glycerol. This muscle was stimulated for fifteen shocks and then frozen during the next fifteen and gave a response like the beginning of tracing 1S, Fig. 1 (normal relaxation). Note that the phosphocreatine is still 39 mg. per cent after stimulation (5S, Fig. 2). 5S', Fig. 2, shows the condition 1 hour after the administration of glycerol and after a comparable stimulation. Note the small amount of phosphocreatine and the degree of dehydration indicated by the apparent increase in total phosphorus. The phosphocreatine was already low in an unstimulated muscle after glycerol as shown by 6U, Fig. 2, and the type of response by its homologue from the same animal is shown by 6S, Fig. 1.

Since dehydration appeared to be the key to one means of producing contracture in frog muscles we tried hypertonic solutions of both glucose and urea. The latter was given in doses of 2 cc. of saturated solution, and the former the same volume of a 75 per cent solution. The effects of both are comparable to those of the other contracture-producing substances (4 and 8, Figs. 1 and 2).

DISCUSSION.

Previous work by others (6, 8, 10, 12) has shown that muscular fatigue is associated with a decrease in phosphocreatine. Fiske (9) has stated that "The hydrolysis of phosphocreatine seems now to be the principal factor permitting contraction to take place to a limited extent without the appearance of fatigue. . . ."

In our work the distribution of the phosphorus compounds studied was the same in the contracture resulting from dehydration as in fatigue contracture. There was also a close relationship

between a low phosphocreatine content and the development of contracture and at the same time there was a large amount of free inorganic phosphate in the muscles.

We endeavored to interpret the results on the basis of exhaustion of the muscles by the repeated excitation from the central nervous system before they were extirpated, since glycerol and bicarbonate produced twitchings and dystonic movements to a marked degree. This explanation does not suffice for glucose and urea since animals given these substances were quiet, yet their extirpated muscles exhibited contracture on stimulation. That the muscles were not exhausted was shown also by their repeated responses to stimulation provided sufficient time was given for relaxation to occur between stimuli.

Dulière and Bouckaert (5) have reported that contractures accompanied by increased acidity in muscle are accompanied by absence of phosphocreatine, but that the alkaline rigor of hypoglycemic death produced by insulin is similarly accompanied by a disappearance of phosphocreatine.

Schwartz and Oschmann (18) found that the A value (phosphocreatine plus inorganic phosphate) was lower in the gastrocnemii of frogs during contracture produced by monobromoacetic acid than in relaxed muscles. Their findings agree with ours and indicate that during fatigue and contracture there is some of the phosphocreatine phosphorus which is not accounted for by the inorganic phosphate set free, but is transferred to the more stable compounds contained between the total value (T) and the A value (4, 6).

Since our work has dealt with a type of contracture which can be produced in the frog by the administration of large doses of hypertonic solutions of physiologic substances, they may not be comparable to contractures produced by drugs such as monobromoacetic acid, chloroform, nicotine, and veratrin. The type of contracture produced by dehydration appears to be readily reproducible, is dependent primarily upon an increase in the relaxation time of the muscle, and is capable of sustaining a greater load than that usually used in the study of drug contractures. Our data do not prove that slow relaxation is invariably associated with a change in the distribution of acid-soluble phosphorus from the normal, but indicate a similarity in the mechanism of production of fatigue and dehydration contracture.

We have been impressed with the similarity of muscular dehydration with its coincident tetany-like symptoms in frogs and the occurrence of tetany in man during disease which causes dehydration. Wechsler (20) lists a number of pathological conditions which are often associated with tetany. Among these are cholera, severe diarrheas in children, pyloric stenosis in infants, and rickets.

SUMMARY.

1. The intraperitoneal injection of hypertonic solutions of sodium phosphates, sodium bicarbonate, glycerol, glucose, or urea produced in frogs an increase in relaxation time of their extirpated gastrocnemii of approximately 5 times the normal.

2. The distribution of inorganic phosphate and phosphocreatine in the muscles from the treated frogs resembled that in muscles from untreated animals during fatigue contracture.

3. Dehydration of the muscle appeared to be the exciting cause of the artificially produced contractures.

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THE COMPOSITION OF THE BODY FLUIDS OF THE GOOSEFISH (LOPHIUS PISCATORIUS).

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The composition of the body fluids of the higher fishes invites attention in view of the unusual nature of these fluids in the more primitive elasmobranchs (Smith (1)). But most of the higher fishes which we have examined have had little or no free fluid in the pericardial or perivisceral cavities. These include one or more specimens of the fresh water carp, goldfish, catfish, sheepshead, bowfin, and gar and the marine puffer, ocean trigger, cod, hake, haddock, flounder, and butterfish.

The presence of fluid in the body cavities of many fishes may be a seasonal phenomenon, for Greene (2) states that fluid is never present in the peritoneal cavity of the male salmon, and is only present in the female when the eggs are being set free.

One apparent exception to this rule, however, is the teleost, *Lophius piscatorius*, both the males and females of which offer sufficient quantities of all these body fluids to permit complete analysis on a single specimen. A relatively small *Lophius*, weighing 10 to 15 kilos, gives 100 to 150 cc. of serum, 10 to 20 cc. of pericardial fluid, 10 to 40 cc. of perivisceral fluid, 10 to 15 cc. of spinal fluid, and 30 to 50 cc. of bile.

The kidneys of this fish have been shown by Audigé (3), Marshall and Grafflin (4), and Edwards (5) to be practically devoid of glomeruli. Whether or not the absence of glomeruli is reflected in peculiarities in the composition of the body fluids, or whether the aglomerular state may be responsible for the continued presence of these fluids, is unknown and must await a more complete examination of other fishes.

Lophius is naturally a water-logged fish, the connective tissue spaces always showing a gelatinous, edematous condition in marked contrast to the majority of fishes. We have not, however, any substantial reason for associating this water plethora with the absence of glomeruli.

As in the elasmobranchs, the pericardial and perivisceral fluids of *Lophius* are colorless, contain only a trace of protein, and have a few leucocytes in suspension.

In removing the body fluids it is customary to expose the pericardium first, by an incision through the pectoral muscles. The bulk of the pericardial fluid must be aspirated with a syringe before cutting the pericardium to prevent loss. The fish can then be bled by aspiration from the large veins near the heart. The perivisceral fluid can be aspirated from the abdominal cavity after penetrating the latter from the ventral surface and moving the viscera to one side. The bile is aspirated from the gallbladder, and the spinal fluid from the brain case after trephining the dorsal surface. In the collection of the latter there is unavoidable but slight hemorrhage.

The methods of analysis were the same as those enumerated in a previous paper (Smith (1)).

Data are given in Table I on the inorganic constituents of the body fluids from several specimens of *Lophius*, and a few additional data are given on other fish. The serum analyses of the ganoids *Amiatus* and *Lepidosteus* are included since such analyses have not heretofore been published.

The analyses on *Lophius*, Fish 1 and 2, were made at the Mount Desert Island Biological Laboratory, Salisbury Cove, in September, 1927. These fish were caught in the open ocean off the Maine coast by local fisherman using trawl or pound nets, and were transported to land and to the laboratory in tubs of sea water. Fish 1 was kept alive for several days by submergence off the laboratory's wharf. Fish 2 was sacrificed soon after it reached the laboratory, the extreme period between capture and sacrifice not exceeding 12 hours. In both instances the fish were alive and apparently normal at the time of removal of the body fluids, and had not been exposed after capture to any other medium than the sea water of the Atlantic Ocean or of Frenchman's Bay.

TABLE I.
Composition of Body Fluids of Lophius piscatorius and Other Fish.

Fish No.		Δ °C.	pH (18°).	mm per liter.							
				Na	K	Ca	Mg	Cl	SO ₄	PO ₄	CO ₃
1	<i>Lophius piscatorius</i> , ♀.										
	Serum.		7.29	201	7.0	3.5	1.0	191	1.2	6.4	6.7
	Pericardial fluid.				7.4	1.8	5.3				8.2
	Bile.		7.72	234	18.2	6.4	2.7	154	0.0	0.0	10.1
2	<i>Lophius piscatorius</i> , ♀.										
	Serum.		7.40	186	7.0*	3.9	15.0	209	3.7	6.7	6.5
	Spinal fluid.		7.62	188	4.0	3.5	10.0	202	3.3	5.1	5.4
	Pericardial fluid.		7.61		4.2	3.4	17.0				7.2
	Perivisceral "		7.60	216	4.7	3.9	16.0	232	8.2	4.7	6.1
	Bile (colorless).		7.82	354	13.1	19.8	3.1	32	†	0.1	7.7
3	<i>Lophius piscatorius</i> , ♂.										
	Pericardial fluid.	0.925		218	5.1	3.0	2.3	207	3.6		14.4
	Perivisceral "	0.892		192	6.9	3.3	2.5	186	3.9		12.9
	Bile.	0.862		244	12.5	13.6	1.2	68	†		22.9
4	<i>Lophius piscatorius</i> , ♂.										
	Serum.	0.840		205	9.0	2.1	1.4	166	3.6	4.9	8.3
	Spinal fluid.	0.838		200	8.8	2.0	2.0	166	3.0	4.3	9.0
	Pericardial fluid.	0.885									10.1
	Perivisceral "	0.990		170	7.5	2.1	2.1	161	2.0	4.1	10.5
	Bile.	0.809									
5	<i>Lophius piscatorius</i> , ♂.										
	Serum.	0.842		202	6.9	2.2		180	2.3	3.2	5.0
	Pericardial fluid.	0.754		176	7.1	2.5		188	2.6		5.4
	Perivisceral "	0.757		178	5.4	2.8		190	2.5		5.8
	Bile.	0.581		178	15.0	6.6		56	†		20.0
6	<i>Gadus callarias</i> .										
	Serum.			180	4.9	5.0	3.8	158		3.1	4.8
	Perivisceral fluid.			165	4.6	6.1	3.1	151		3.3	11.0
	Pericardial "			163	4.7	5.1	1.0	169			7.7
7	<i>Opsanus tau</i> .										
	Serum.										11.2
	Perivisceral fluid.										13.5
8	<i>Spheroides maculatus</i> .										
	Serum.				3.2	8.3	4.5	162	1.0	5.3	8.0
	Pericardial fluid.										10.1
9	<i>Amiatus calva</i> , ♀.†										
	Serum.	0.530		132	1.7	6.0	0.4	120	1.4	4.5	4.5
10	<i>Amiatus calva</i> , ♀.†										
	Serum.	0.540		133	2.3	4.6	0.4	119	3.0	3.7	4.1
	Pericardial fluid.										4.1
11	<i>Lepidosteus osseus</i> .†										
	Serum.	0.570		140	2.7	6.1	0.3	118	2.4	3.9	9.1

* Slight hemolysis. † Less than 1.0. ‡ Not eating in winter time.

Fish 3 to 5 (*Lophius*) were caught in pound nets off Sandy Hook by the fishing steamer of the New York Aquarium, between October 10 and 27, 1928. They were brought to the Aquarium in tubs of sea water, where, as is customary with *Lophius*, they were placed in tanks through which the local Harbor water is circulated. At this particular time, the Harbor water had a freezing point of -1.1° . *Lophius*, in the experience of the Aquarium, does not live well in captivity, apparently because it cannot tolerate warm water. All the sea water in the Aquarium is warmed, and since this fish is known to migrate naturally into New York Harbor, it is thought that the lower salinity of the Harbor water is less deleterious than the warmer sea water. But even the Harbor water is too warm, or otherwise unsuitable, for apparently healthy and vigorous specimens rarely survive more than 2 weeks.

The other fish were obtained through the courtesy of the New York Aquarium.

DISCUSSION.

There is no marked and consistent inequality in the distribution of K, Ca, Mg, Cl, or SO_4 , in the various body fluids of *Lophius* as there is in the eusmibranchs. Except for the smaller quantity of protein in them, the spinal fluid, perivisceral fluid, and pericardial fluid closely resemble serum. With a few exceptions, these fluids might be considered as simple dialysates formed by the diffusion of plasma across indifferent membranes which restrained only the plasma proteins.

The Mg in Fish 2 is extraordinarily high in comparison with the other specimens; so high, in fact, as to suggest pathological retention.

One noteworthy feature is the somewhat higher CO_2 content of the pericardial and perivisceral fluids of *Lophius* as compared with the serum. In the few instances where pericardial or perivisceral fluids have been present in the other fishes examined, these have shown a CO_2 content equal or slightly greater than that of the serum. It may be said that the CO_2 analysis of these fluids was carried out with greatest precaution against loss of CO_2 , though the small quantities of fluid present and the conditions under which the samples must be taken make the determination difficult.

If it is assumed that the same CO_2 tension prevails in the serum and in these body fluids, this higher CO_2 content indicates that these fluids are normally more alkaline than the plasma, a condition the reverse of that existing in the elasmobranchs. Whether or not this condition is typical of teleost fishes would require a more protracted examination than is possible for us to carry out at the present time.

The present data suggest that such may be the case, and they are particularly interesting in view of the extreme alkalinity prevailing in these body fluids in the Amphibia and reptiles, on which a report will be made in the future.

SUMMARY.

In inorganic composition, the spinal, pericardial, and perivisceral fluids of *Lophius piscatorius* show no consistent or emphatic deviations from the serum.

There is some evidence that the pericardial and perivisceral fluids of *Lophius* and other teleosts may be normally slightly more alkaline than the plasma.

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AMINO ACID CATABOLISM.

III. THE FATE OF THE ω -HYDROXY DERIVATIVES OF PROPIONIC, BUTYRIC, VALERIC, AND CAPROIC ACIDS IN THE PHLORHIZINIZED DOG.*

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In an earlier communication there was presented the hypothesis that one of the paths of catabolism of the diamino acids might be through the stage of the ω -amino acids having 1 less carbon atom (Corley, 1926). As evidence for this there were the findings that γ -aminobutyric acid like ornithine was, while δ -aminovaleric acid like lysine was not a sugar former in the diabetic organism. Recently a report has been made to show that β -alanine and ϵ -aminocaproic acid are not sugar formers in the completely phlorhizinized dog (Corley, 1929). In view of the numerous observations that amino acids may be converted to the corresponding hydroxy derivatives in the animal body, there arises the possibility that one of the paths of breakdown of the ω -amino acids is by the way of the ω -hydroxy acids. As a check of this relationship, it has seemed of interest to investigate the fate of the latter in the diabetic animal. In addition there may be obtained evidence in regard to the effect on metabolism of a hydroxy group in other than the α position.

Fasting female dogs have been treated with purified phlorhizin until there has been established a constant ratio between the glucose and nitrogen excreted in the urine. Except as indicated below, the urine has been collected during 24 hour intervals, each ending in the early forenoon. An attempt has been made to

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collect all urine by catheter, particularly during the days when extra sugar formation was studied.

In view of the fact that these substances could be expected to affect directly only one of the values, that of glucose in the quotient of glucose to nitrogen, it has seemed possible and desirable to collect two samples of urine on the day that administration of the compound has been made. This has the obvious advantage that smaller amounts of additional sugar can induce more marked alterations in the G:N ratio. The acids have therefore been administered early in the experimental day, and a urine sample collected for the first 12 hours, and another for the second 12

TABLE I.
Sugar Formation from Hydracrylic Acid.

Dog 3.

Day of fasting.	Weight.	Urinary glucose.	Urinary nitrogen.	G:N
	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	
7	9.50	33.77	10.08	3.35
8	9.30	34.94	10.55	3.31
9	9.05	32.26	9.80	3.29
10	8.85	31.70	9.71	3.28
11	8.60	28.06	8.06	3.48

1.2 gm. of phlorhizin were given daily beginning the 2nd day of fasting. 6 gm. of hydracrylic acid in molecular Na_2CO_3 solution were administered subcutaneously in two doses at 9.45 a.m. and 2.45 p.m. on the 10th day. The non-protein nitrogen of the blood was 30 mg. per cent the previous day; 32 mg. per cent the following day. Extra glucose, none.

hours. Whereas it has not seemed necessary to make this subdivision on other days, the G:N ratios of the preceding day and the following day have been given double the weight of that of the last half of the experimental day in the calculation of the prevailing G:N ratio. The difference between this value and that of the period of the administration of the compound studied, times the nitrogen of this period has been taken to be the extra glucose formed.

Three of the substances studied have been synthesized in the laboratory of the Department of Chemistry of the University of Illinois. In view of the difficulty of isolating the free acids, recourse has been taken to their separation as the sodium salts.

Marvel and Birkhimer (1929) have made a report of the preparation and analysis of these, the sodium salts of the ω -hydroxy derivatives of butyric, valeric, and caproic acids. β -Hydroxypropionic, technical hydracrylic acid, was obtained from the Eastman Kodak Company and employed without attempts of further purification. The results with this compound as a consequence are open to criticism and would be particularly so if

TABLE II.
Sugar Formation from γ -Hydroxybutyric Acid.

Dog 2.					Dog 11.				
Day of fasting.	Weight.	Urinary glucose.	Urinary nitrogen.	G:N	Day of fasting.	Weight.	Urinary glucose.	Urinary nitrogen.	G:N
	kg.	gm.	gm.			kg.	gm.	gm.	
4	7.25	24.62	6.29	3.91	5	7.13	22.04	6.78	3.25
5	6.80	18.40	4.91	3.75	6	6.92	20.92	6.64	3.15
6	6.58				7	6.81			
0-12 hrs.		9.44	2.31	4.09	0-13 hrs.		13.12	3.53	3.71
12-24 "		6.82	1.89	3.61	13-24 "		8.82	2.91	3.03
7	6.35	12.99	3.84	3.38	8	6.48	16.62	5.38	3.09

Dog 2.—0.7 gm. of phlorhizin was given daily beginning the 2nd day of fasting. 3.5 gm. of sodium γ -hydroxy butyrate in 20 per cent aqueous solution were administered subcutaneously in two doses at 10.20 a.m. and 2.40 p.m. on the 6th day. The non-protein nitrogen of blood was 30 mg. per cent the previous day; 27 mg. per cent the following day. Extra glucose 1.20 gm.

Dog 11.—0.8 gm. of phlorhizin daily beginning on the 2nd day. 4 gm. of sodium γ -hydroxy butyrate in 20 per cent aqueous solution were administered subcutaneously in two doses at 8.50 a.m. and 1.10 p.m. on the 7th day. The non-protein nitrogen of the blood was 36 mg. per cent the previous day; 36 mg. per cent the following day. Extra glucose 2.17 gm.

they indicated gluconeogenesis. Since, however, there was no extra sugar formation, it is felt not unreasonable to accept the results as presumptive evidence in the problem at hand.

With the exception of the butyric acid derivative, these ω -hydroxy acids seemed more toxic than the corresponding amino acids. For this reason, some of the experiments proved rather unsatisfactory for purposes of interpretation. In these, nitrogen retention, shown either by the non-protein nitrogen figures of the

blood or by decided drops in the daily nitrogen excretion, have caused the G:N ratio to rise. It may be said, however, that the increases did not occur until subsequent to the crucial period, the

TABLE III.
Sugar Formation from δ -Hydroxyvaleric Acid.

Dog 10.

Day of fasting.	Weight.	Urinary glucose.	Urinary nitrogen.	G N
	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	
5	8.15	25.72	8.01	3.21
6	7.70	26.24	7.90	3.32
7	7.45			
0-12 hrs.		13.51	4.05	3.33
12-24 "		10.32	3.30	3.12
8	7.25	19.32	5.82	3.32

0.9 gm. of phlorhizin was given daily beginning the 2nd day of fasting. 3 gm. of sodium δ -hydroxy valerate in 20 per cent aqueous solution were administered subcutaneously in two doses at 8.35 a.m. and 1.50 p.m. on the 7th day. The non-protein nitrogen of the blood was 35 mg. per cent the previous day; 30 mg. per cent the following day. Extra glucose, none.

TABLE IV.
Sugar Formation from ϵ -Hydroxycaproic Acid.

Dog 14.

Day of fasting.	Weight.	Urinary glucose.	Urinary nitrogen.	G N
	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	
4	12.35	23.90	7.39	3.23
5	12.20	19.91	6.57	3.03
6	11.90			
0-12 hrs.		13.47	4.77	2.82
12-24 "		12.31	4.33	2.84
7	11.80	26.12	8.69	3.00

1.2 gm. of phlorhizin were given daily beginning the 2nd day of fasting. 3.50 gm. of sodium ϵ -hydroxy caproate in 2.0 per cent aqueous solution were administered subcutaneously in two doses at 9.45 a.m. and 2.20 p.m. on the 6th day. The non-protein nitrogen of the blood was 35 mg. per cent the previous day; 28 mg. per cent the following day. Extra glucose, none.

one during which the compound was administered. As a matter of fact the change in the G:N ratio even in these cases has been rather gradual, so that calculations made in the customary manner

have furnished indications corresponding to these obtained from more acceptable experiments, but it is not felt that conclusions made from such protocols are above criticism. It has, however, been possible to obtain at least one satisfactory experiment with each of the compounds employed.

Of the derivatives only that of butyric acid has given evidence of gluconeogenesis. In three experiments, 11 gm. of sodium γ -hydroxy butyrate with glucose equivalents of 5.24 gm. calculated for 2 carbon atoms or 7.85 gm. calculated for 3 carbon atoms, caused the production of 6.31 gm. of extra sugar. The conclusion seems warranted, therefore, that γ -hydroxybutyric acid is a sugar former in the completely phlorhizinized dog.

The results in this study with hydroxy acids are strikingly similar to those obtained with the corresponding amino acids. The derivatives of butyric acid are gluconeogenetic, while those of propionic, valeric, and caproic acids are not. Dakin (1922, p. 142) has stated that "in general it will be noted that the behavior of the simple primary amines in the body resembles that of the corresponding alcohols and acids." It appears not unlikely that with increasing distance from the carboxyl group an amino group in an acid may assume more and more the physiological properties of an amino group in an amine.

There has been an impressive accumulation of evidence that the fate of α -amino acids and α -hydroxy acids is the same. That phenyl serine and phenyl glyceric acid are both oxidized to benzoic acid in the animal body (Dakin, 1909, *b*) may be taken to indicate that further substitution may not affect this relationship. Mayer (1904) has shown that glyceric acid appears in the urine when α , β -diaminopropionic acid is injected, demonstrating the ability of the body to replace with a hydroxy group an amino group in other than the α position. Furthermore, when phenyl- β -alanine has been administered to animals, phenyl- β -hydroxypropionic acid has been recovered from the urine (Dakin, 1909, *a*). Most β -hydroxy derivatives are apparently degraded further with facility.

The observations on the fate of compounds with hydroxy groups in other than the α or β position have been rather infrequent.

Dakin (1908-09) found that phenyl- β , γ -dihydroxybutyric acid was changed to benzoic acid as if γ -oxidation had occurred, but,

on the other hand, that when he administered phenyl- γ -hydroxyvaleric acid to a cat, a large portion was recovered and no hippuric acid or other products were detected in the urine (1909, a).

Ringer, Frankel, and Jonas (1913) have made the suggestion that the oxidation of the diamino acids proceeds in such a way as to give the dicarboxylic acids having 1 less carbon atom. The results obtained in this series of studies present evidence as to the mechanism of such a course of metabolism. It is felt that the data support the view that successive stages in the breakdown of the diamino acids may be the ω -amino acids resulting from the loss of 1 carbon atom, the corresponding ω -hydroxy acids, and the related dicarboxylic acids. The evidence for this latter step is not direct. There is of course the correlation in the ability to form extra sugar possessed by ornithine and succinic acid, and the lack of such ability for lysine and glutaric acid. Luzzato (1905-06) found that after the administration of sodium hydracrylate there were practically no ether-soluble derivatives in the urine. In one case only, were there detected traces of an acid resembling malonic acid.

SUMMARY.

1. The results obtained have been interpreted to indicate that γ -hydroxybutyric acid is a sugar former in the completely phlorhizinized dog, while hydracrylic acid, δ -hydroxyvaleric acid, and ϵ -hydroxycaproic acid are not.

2. It is felt that the data obtained in this series of studies support the view that successive stages in the breakdown of the diamino acids may result in the acids with 1 less carbon atom, with an amino group in the ω position, the corresponding ω -hydroxy acids, and the corresponding dicarboxylic acids.

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TWO REVISED COPPER METHODS FOR BLOOD SUGAR DETERMINATION.

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In this paper are given the results obtained in the course of a critical review of the 1926 copper method for the determination of sugar in blood (1).

When 0.1 mg. of glucose (in 2 cc.) is determined, using the regular standard (0.2 mg. in 2 cc.), one obtains colorimeter readings of about 20:43.5 instead of the theoretical 20:40 mm. This unsatisfactory range of proportionality cannot be materially improved by altering the composition of the copper reagent. On the contrary, the 1926 copper solution seems to represent nearly as perfect a mixture of copper tartrate and carbonates as it is possible to make for the oxidation of small amounts of glucose in pure, approximately neutral solutions. The molybdate solution used in this method for the oxidation of the cuprous oxide is also one which can scarcely be improved. The lack of a satisfactory range of true proportionality has now been found to be due to a more or less incorrect way of using the method. There is some loss of color and also an unmistakable change of shade when the blue solution is diluted from 6 to 25 cc. This change, which continues after the dilution, is more pronounced in the weaker solutions, and this is the reason why the weaker solutions give too little color. By using dilute molybdate reagent instead of water for the dilution to the 25 cc. mark, the error is corrected and substantially theoretical readings of 20:40 are obtained for 0.2 and 0.1 mg. of glucose. For 0.2 and 0.4 mg. of glucose the colorimetric reading will be 20:10.2 or 10.3. More than 0.4 mg. of glucose, therefore, must not be determined by means of this copper reagent.

After the discovery of this simple improvement in the use of the

molybdate reagent it seemed expedient to study the Folin-Wu method from the same point of view. This method, as is well known, tends to give values that are too high for the weaker glucose solutions, so much so that tables of corrections have been published and are often used. These extra sugar values are due to the fact that the Folin-Wu copper reagent always gives some color when the molybdate reagent is added to a heated mixture of 2 cc. of water and 2 cc. of the copper reagent. This blank has always been considered very small. Everett and Sheppard (2) have recently determined its value and have found that it is equivalent to about 5 mg. per cent of blood sugar. But the problem is not quite so simple. Freshly prepared Folin-Wu copper reagents give extremely little color in blank experiments. If with such a flawless reagent 0.1 mg. of glucose is determined by the help of a standard containing 0.2 mg., the colorimeter readings will be about 42.3:20 when the molybdate reagent is used as described above, and the error will be greater if the dilution is made with water in the usual way. The actual blank with old Folin-Wu copper reagents, therefore, must be materially larger than the value found by Everett and Sheppard.

Because of the new conditions which have come in from the use of insulin, it is more important to be able to obtain reasonably correct blood sugar values in hypoglycemia than in hyperglycemia. It is, therefore, more important to obtain true proportionality over the range of 20:40 mm., than over the range of 20:10. It would seem, however, that true proportionality over the range 20:40 is obtainable only at or near the weak alkalinity represented by the 1926 reagent.

In the light of these observations it seems necessary to describe again the 1926 copper method for blood sugar as we now use it, and to give such additional information as may be available.

1. On Tartrates as a Constituent of Alkaline Copper Solutions.

Since practically every one is now using tartrates for alkaline copper solutions which are employed for the determination of small amounts of sugar, it seems desirable that fuller knowledge than is yet available should be obtained concerning the behavior and function of this ingredient. I shall, therefore, give here the few observations which were incidentally obtained in this work.

With freshly prepared copper reagents, very much smaller amounts of tartrate than are commonly used are sufficient to hold the copper in solution. The greater the amount of tartrate present, the greater is the tendency to get autoreductions at room temperatures, and to get further reductions (blanks) on heating. With freshly prepared reagents the formation of perceptible blanks begins at a sodium tartrate content of about 11 or 12 gm. per liter in the presence of 5 gm. of copper sulfate. With fresh solutions the blanks are not much greater at an alkalinity of the Folin-Wu reagent than at that of the Folin solution,—at least below 15 gm. of sodium tartrate per liter. But the more alkaline solutions deteriorate much more rapidly and then give larger blanks. This deterioration speeds up with the temperature. In a refrigerator, even the Folin-Wu reagents keep for some time without any *visible* formation of cuprous oxide.

The most conspicuous defect of tartrates in the alkaline copper reagents is the autoreduction. If this were the only effect of the tartrates, in addition to holding the copper in solution, the obvious remedy would be to use very small amounts, but the tartrates also modify the course of the reaction. At the alkalinity of the 1926 reagent, the tartrates retard and inhibit the reduction. For each gm. of sodium tartrate above 7 gm. per liter there is a demonstrable increase in the length of time required for the first appearance of turbidity; and for each gm. above 10 gm. per liter, there is also a loss of about 5 per cent in the amount of cuprous oxide obtainable from 0.2 mg. of glucose (by 10 minutes heating). This fact would seem to suggest again that the best sodium tartrate content should be very low, between 7 and 10 gm. per liter. At the alkalinity of this reagent it is impossible, however, to secure perfect proportionality between 0.2 and 0.1 mg. of glucose with a tartrate content of less than 11 gm. per liter. And 12 gm., the original tartrate content of the reagent, is a shade better than 11 gm.

2. Deterioration of the Copper Reagent.

Closely associated with the tartrate content of the reagents is the problem of the keeping quality. In the Folin-Svedberg (3) paper the deterioration was ascribed to an increase of alkalinity by the spontaneous loss of carbon dioxide. Benedict (4) rejected

this interpretation and ascribed the loss to oxidation of the tartrate by atmospheric oxygen. Since this reagent is very finely adjusted both as regards alkalinity and tartrate contents, either interpretation, if correct, might be adequate to account for the deterioration. And if either or both explanations were substantially correct the remedy suggested by Folin and Svedberg—to keep the reagent in well filled, small, tightly corked, bottles—would seem a fairly good practical solution. A small bottle, once opened, then, of course, would not be used very long. Benedict's alternative remedy of keeping the reagent in the form of two separate solutions is the good old remedy that all of us used long ago with Fehling's solutions. But in this case it is not theoretically clear that the old remedy would work. If the tartrate were oxidized by atmospheric oxygen it might be oxidized just as well in the absence as in the presence of the copper, and if loss of CO_2 were the responsible factor, this also would not be prevented by keeping the copper in a separate solution.

Any one can easily satisfy himself that sodium bicarbonate solutions readily lose CO_2 . Dissolve 10 gm. of the bicarbonate in 500 cc. of water and leave the solution in an unstoppered Florence flask (capacity 1 liter). At the end of 24 hours dissolve another 10 gm. of the bicarbonate in 500 cc. of water. Add 1 cc. of phenolphthalein solution to each. The older solution turns deep red, and it will require the addition of at least 5 cc. of 10 per cent sodium carbonate to produce as deep a color in the freshly prepared solution.

On the other hand, heating 4 cc. of a 1 per cent solution of sodium bicarbonate in a Folin-Wu sugar tube for 10 minutes seems to produce very little loss of CO_2 .

I have satisfied myself, however, that Benedict is correct, that decomposition of the tartrate rather than loss of carbon dioxide is the cause of the deterioration in all alkaline copper tartrate solutions, including those most weakly alkaline, and that no tartrate decomposition or appreciable losses of CO_2 (in reasonably well handled bottles) take place in the absence of the copper.

3. Time of Heating.

While it is desirable to secure complete decomposition of the sugar and the maximum intensity of color in colorimetric sugar determinations, the most important consideration is true and

dependable proportionality between widely different amounts of sugar. Complete destruction of the sugar is most readily obtained with the more alkaline solutions, but these give neither the maximum color nor true proportionality. The heating time originally selected for the Folin method was 10 minutes, because this gave substantially correct proportionality between 0.2 and 0.4 mg. of glucose. The error introduced by diluting with water helped to make this proportionality more perfect than it should have been.

A 10 minute heating in rapidly boiling water still gives nearly correct proportionality between 0.2 mg. and 0.4 or 0.1 mg. of glucose, but by heating 12 or 15 minutes one obtains a slight but unmistakable improvement in proportionality and obtains about 20 per cent more color. Even after 15 minutes heating one does not obtain quite the maximum amount of cuprous oxide, but it is not safe to heat much longer than 15 minutes, because of possible autoreduction by the tartrate. In the presence of much tartrate, or much copper, one does not get to even an approximate endpoint, because the reduction from the tartrate then overlaps the reduction from the sugar.

4. *Preparation of the Copper Reagent.*

1. *Alkaline Tartrate Solution.*—Transfer 7.7 gm. of anhydrous sodium carbonate to a volumetric liter flask, add from 50 to 75 cc. of distilled water, and shake for a few moments. The carbonate dissolves more quickly in a little water than in a large volume because of the generated heat. Wipe the mouth of the flask dry and then introduce into the flask 22 gm. of sodium bicarbonate. Rinse the bicarbonate from the neck into the bottom of the flask with enough distilled water to give a volume of 700 to 800 cc., and shake. The bicarbonate dissolves at once. Finally introduce 13.2 gm. of Merck's c.p. sodium tartrate, rinse the neck of the flask, and shake until complete solution is obtained. Dilute to volume, mix, and keep in a tightly stoppered bottle or flask.

2.—A 5 per cent solution of c.p. crystallized copper sulfate to which has been added a trace of concentrated sulfuric acid to prevent the formation of a copper sediment.

3. *Alkaline Copper Tartrate Solution.*—Pour some of the tartrate solution (25 cc.) into a 50 cc. volumetric flask, with a pipette add 5 cc. of the copper sulfate solution, and dilute to volume with

the tartrate solution. Mix. In a refrigerator this solution will keep for some days, but at room temperatures, particularly in hot weather, or when exposed to direct sunlight, it may deteriorate in the course of a few hours.

Only the best C.P. grades of chemicals should be used in the preparation of this reagent. It is probably not possible to purchase any strictly pure tartrates. They contain a small amount of impurity which in the course of 10 to 15 minutes gives some color with the molybdate reagent. The color thus obtained from 2 cc. of copper reagent containing no more than 12 gm. of sodium tartrate per liter is, however, very slight indeed.

5. Acid Molybdate Reagent.

A rapid method for preparing this reagent is described in the original paper. The only defect of the reagent so prepared is that it will turn somewhat blue in the course of a few days. This defect will disappear whenever purer grades of sodium molybdate become available. Even as it is, the preparation of this reagent twice a week is not much of a drawback, and the reagent should keep much longer in a refrigerator. The process of preparing this temporary reagent is as follows: Dissolve 40 gm. of sodium molybdate in 100 cc. of distilled water in a 500 cc. beaker. The molybdate dissolves extremely quickly (2 to 3 minutes) but a certain turbidity is left which does not clear up. To the turbid solution add, with stirring, 55 cc. of 85 per cent phosphoric acid, 40 cc. of cool sulfuric acid (25 per cent, 1 volume of H_2SO_4 to 3 volumes of water) and finally 20 cc. of 99 per cent acetic acid. The resulting mixture is at once ready for use.

For the preparation of the purified reagent it is advantageous to keep on hand a brominated 30 per cent solution of sodium molybdate. By means of a large funnel and fine glass rod transfer 600 gm. of sodium molybdate to a 2 liter volumetric flask. Add much water and shake until solution is complete except for the turbidity. Dilute to volume, mix, and transfer this stock solution to a large flask or bottle. Add about 0.5 cc. of liquid bromine, shake, and set aside. Transfer 500 cc. of the clear supernatant solution to a Florence flask, capacity 1000 to 1500 cc. Add with stirring 225 cc. of 85 per cent phosphoric acid. Some bromine is set free and imparts a yellow color to the solution. Next add

150 cc. of cool sulfuric acid (25 volumes per cent). Remove the bromine by means of an air current either immediately while the solution is still warm or better still the next day. Then add 75 cc. of 99 per cent acetic acid. Mix and dilute to 1 liter. If protected from organic matter this reagent will remain colorless for months.

6. Use of the Colorimeter in Sugar Determinations.

For many years now I have used only Bausch and Lomb colorimeters with colorimeter cups enclosed in a metal frame. Many workers do not seem to understand the advantages of these cups and the action of the molybdate reagent on the metal in connection with sugar determinations has served to emphasize the one serious defect which these cups undeniably have; namely, the escape of the colored fluids into the space between the glass and the metal.

These cups were devised to remedy a defect nearly always present in the French instrument which was earlier used. In these instruments the zero point was seldom correct and the two cups were not interchangeable, so that one had to remember on which side each cup belonged in order to preserve correct zero points. In addition, the glass cups became loose now and then and had to be reset,—a difficult task from the standpoint of the zero point. In the Bausch and Lomb cups with metal frames the zero point is easily adjusted by screwing the bottom against the rubber ring. A supply of such rubber rings may be had from the manufacturers, but each ring usually lasts for months. If a new ring proves to be a little too thick, it can be filed thinner by means of a large, coarse, flat file. When the cups have once been adjusted, the bottom is left undisturbed for months at a time. All of the liquid between the glass and the metal is washed out by leaving them for a few minutes under running water. But the escape of the colored fluids into the space between the glass and the metal is entirely prevented by packing the space near the top of the cups with a little stop-cock grease (1 part of vaseline to 3 parts of ordinary paraffin). It is best to have both the cups and the grease a little warm for this packing. There is no pressure, as in burettes, and the grease excludes the liquids perfectly, if the cups are rinsed only with cold water.

When thus packed, the cups will function like cups made only of glass, except for the action of the acid molybdate solution on the metal rim. Different kinds of lacquer and platings have been tried but none has proved effective, and all of these are soon worn off the rim by the constant wiping. This defect probably has to be accepted as the price for the many advantages which these cups have,—not the least of which is that they almost never break, even in the hands of students. The defect becomes noticeable practically only in connection with sugar determinations by copper methods. And even this disadvantage can be made to vanish very nearly by the simple device of daily rubbing the rims with a piece of beeswax.

One simple elementary rule in colorimeter work is that not much more liquid than is needed shall be poured into each cup. Only beginners are expected to pour in so much that spilling takes place when the plunger is lowered. With such careless work in sugar determinations the acid liquid can even reach the metal into which the plungers are cemented. It is easier to rinse off the plungers if they have not been immersed too far into the colored liquids. One can tell, of course, by inspection about how much liquid is needed in each cup.

7. Standard Glucose Solutions.

After several years of experience with benzoic acid as a preservative for glucose solutions, I remain convinced that it is perfectly reliable. The most concentrated stock solution should be made from saturated or nearly saturated benzoic acid solution. This should contain 10 mg. of glucose per cc. It will keep indefinitely, for years.

From the concentrated stock solution prepare an intermediate stock solution containing 2 mg. of glucose per cc. This solution will also keep indefinitely.

. From the stock solution containing 2 mg. of glucose per cc., prepare the working standard solutions containing 0.1 and 0.2 mg. of glucose per cc., again by simple dilution with water. A few drops of formalin or of toluene per liter of this solution should be added to it if it is to be kept a long time, but I prefer to add nothing and to make fresh standards a couple of times a week. The need for preservatives seems to depend very much on what kind of work is done in a given laboratory.

8. *Revised Copper Methods of Folin and of Folin and Wu for Determination of Sugar in Blood.*

Folin Method.—Transfer 2 cc. of tungstic acid blood filtrate to a Folin-Wu sugar tube, or take 1 cc. if not less than 200 mg. per cent of blood sugar are expected. From a dropping bottle or with a pipette add 1 drop of dilute phenolphthalein solution. The phenolphthalein solution *must be diluted* (0.1 per cent). By means of a pipette or a glass tube having a fine point add 1 per cent sodium carbonate solution, drop by drop, until a *permanent* red color is obtained. Transfer 2 cc. of a sugar standard containing 0.1 mg. of glucose per cc. to another sugar tube. If desired this standard can also be treated with phenolphthalein and sodium carbonate solution (1 drop). Add 2 cc. of freshly mixed copper tartrate reagent to each tube and heat in rapidly boiling water for 14 to 15 minutes. Cool in running water.

Add 4 cc. of the acid molybdate reagent to each and after about 1 minute dilute to volume, *not with water*, but with diluted acid molybdate reagent (1 volume of the reagent mixed with 4 volumes of water). Mix and make the color comparison, not omitting first to make sure that the fields in the colorimeter look alike when the standard solution is in both cups and both sides are set at 20 mm.

It is quicker and more efficacious thus to adjust the colorimeter and the eye than to read the standard in one of the cups as if it were unknown. After such a preliminary adjustment I take only one careful reading of the unknown.

Folin-Wu Method.—It is really just as important with this method as with the Folin method that only freshly mixed copper tartrate reagent be used. The reagents can be made up as follows:

(a) Transfer 35 gm. of anhydrous sodium carbonate to a volumetric liter flask, add 175 to 200 cc. of water, and shake for a few moments. Then add 13 gm. of sodium tartrate and 11 gm. of sodium bicarbonate. Add water to a volume of about 800 cc., and shake until clear solution is obtained. Dilute to volume and mix.

(b) A 5 per cent copper sulfate solution acidified with a trace of sulfuric acid. This is the same copper solution as is used in the Folin method.

(c) The working reagent is prepared in the same way. Half fill a 50 cc. volumetric flask with the alkaline tartrate solution (a). Add 5 cc. of the 5 per cent copper sulfate solution, dilute to volume with the tartrate solution, and mix.

The Determination.—Transfer 2 cc. of blood filtrate to a Folin-Wu sugar tube, or 1 cc. plus 1 cc. of water if very high blood sugar values are expected. Transfer 2 cc. of the sugar standard (0.1 mg. glucose per cc.) to another sugar tube. Add 2 cc. of freshly mixed copper tartrate reagent to each tube and heat in rapidly boiling water for 8 minutes. Cool in running water. Add 4 cc. of the acid molybdate reagent and after waiting about 1 minute dilute

TABLE I.

Showing Deviations from True Proportionality Obtained by Revised Folin-Wu Method. Standard Was 0.2 Mg. with Colorimeter Set at 20 Mm.

Glucose taken.	Colorimeter readings.		Difference to be added or subtracted.
	Found.	Theoretical.	
<i>mg.</i>	<i>mm.</i>	<i>mm.</i>	
0.10	42.25	40.00	-2.25
0.13	31.30	30.77	-0.53
0.15	26.93	26.66	-0.27
0.18	22.26	22.22	-0.04
0.20	20	20	0
0.24	16.60	16.66	+0.06
0.30	13.26	13.33	+0.07
0.36	10.83	11.11	+0.28
0.40	9.56	10.00	+0.44

to volume with diluted acid molybdate reagent, mix, and make the color comparison.

As stated in a previous section, the proportionality obtainable with the Folin-Wu method is not so good as with the Folin method, and when made as described here the proportionality obtained for widely different amounts of sugar is quite different from the proportionality obtained with the original method. The weaker solutions are now too weak, whereas formerly they were too strong. Dr. A. D. Marenzi has carefully determined in triplicate the colorimetric readings obtainable from different amounts of glucose between 0.1 and 0.4 mg. when compared against the 0.2 mg. standard. These figures, given in Table I, illustrate the

deviations from true proportionality which are obtained. The figures incidentally give the required data for calculating the correct values obtainable by this method.

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THE NEPHROPATHOGENIC ACTION OF CYSTINE.

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INTRODUCTION.

Preliminary to some investigations previously reported from this laboratory, fatalities occurred among young white rats of 40 to 50 gm. body weight, when restricted to synthetic diets containing 0.3 per cent of free cystine. When placed upon the ration, the animals ate eagerly and grew normally for about 10 days. Then some of them lost their appetites completely, appeared drowsy, and assumed a crouched position. After a day or two in this condition many of them died, and at autopsy their kidneys were found to be very much enlarged and congested. Attempts to save the rats by restoring the stock ration were apparently successful although a few died after this change and all continued to lose weight for several days. Then a rapid recovery took place among the survivors and after a few days they were apparently entirely normal. The malady seemed to vary in its intensity in different litters. Complete change of all food materials, particularly of the ingredients of the salt mixture, did not eliminate the trouble. Similar symptoms were never observed in rats of 80 to 90 gm. in body weight.

Subsequent to the above observations, Lewis (1925) reported that a severe nephritis resulted in rabbits following the oral administration of 1 to 4 gm. of cystine. Although Lewis employed dosages relatively larger than our rats were ingesting, nevertheless his findings suggested that the free cystine of our ration was the causative nephrotoxic factor.

Other investigations of a possible nephrotoxic action of cystine have been reported recently. Newburgh, Marsh, Clarkson, and Curtis (1925) fed rats rations containing 8 per cent of casein and added cystine varying from 0 to 1.25 per cent. They apparently started their experiments when the rats were 30 days old. They found that rats receiving more than 0.75

per cent of cystine show albumin and casts only after 90 days. Curtis and Newburgh (1926) report that large doses of cystine administered to rats cause a hemorrhagic nephropathy and death within a few days. Addis, MacKay, and MacKay (1926-27) fed rats on diets high in protein and cystine. Their control diet was not very different from ours. The high cystine ration was prepared by adding 1 gm. of cystine to 99 gm. of the control food. They observed no differences between the control rats and those upon the high cystine diet. Their rats, being at the start of the experiment 30 days old and weighing about 60 gm., were at the upper limit of the critical period that we have found. Curtis and Newburgh (1927) report mild renal injuries in rats on a 0.5 per cent cystine ration. Over 5 per cent of cystine in the diet resulted in death in less than 10 days for rats of varying ages. Abderhalden (1922) has observed renal injuries in rats ingesting free cystine or tyrosine with crystals of these insoluble amino acids in the tubules.

Newburgh and Curtis (1927), studying the nephropathogenic effect on rats of diets very rich in casein, beef protein, or lactalbumin, were able to keep the animals over long periods on either of the first two proteins. In the case of lactalbumin they report that "11 young rats that ingested the usual amounts of diet containing 75 per cent of lactalbumin, died, without exception in 7 days or less." The toxicity of the lactalbumin may be due to its high content of cystine. Jones, Gersdorff, and Moeller (1924-25) report finding about 4 per cent of cystine in lactalbumin, the highest percentage found in any of the food proteins examined by them.

EXPERIMENTAL.

To test whether the free cystine of our diets was the cause of the acute nephritis observed in our young rats, the following preliminary experiment was conducted. Two groups of young rats from the same litter, each weighing 50 gm. or less, were fed *ad libitum* on the diets shown in Table I.

The analytically pure cystine was prepared in this laboratory and had a specific rotation of -206.0° (Andrews, 1925). The salt mixture was that of Osborne and Mendel (1919). The other materials were commercial products. 50 mg. of Yeast Vitamine (Harris) were supplied daily in the form of a pill made up with dextrin and water. The animals were caged separately and cared for by essentially the methods of Osborne and Mendel (Ferry, 1919-20).

The growth and food consumption of four rats in this preliminary experiment are recorded in Table II. The rats, initially 19 days old, were killed at the end of the 16 days on the above diets and their kidneys examined. The kidneys of Rats 253 and

255, which had not received free cystine, were entirely normal and healthy in appearance; those of Rat 256 were enlarged and slightly congested; those of Rat 254 were white and mottled with hemorrhagic spots and very much swollen. The previously observed complete loss of appetite was not noted, but the decline in weight is quite marked in the case of Rat 254.

TABLE I.
Composition of Diets.

	Diet C.	Diet 6.
	<i>per cent</i>	<i>per cent</i>
Casein.....	15 0	14 4
Cystine.....	0 0	0.6
Dextrin.....	40 0	40 0
Sucrose.....	15 0	15 0
Lard.....	19 0	19 0
Cod liver oil.....	5 0	5 0
Salt mixture.*.....	4 0	4 0
Agar agar.....	2 0	2 0

* Osborne and Mendel (1919).

TABLE II.
Growth and Food Consumption of Rats on (1) Casein and (2) Casein Plus Cystine Diets.

Days.	Rat 253♂. Diet C.		Rat 254♂. Diet 6.		Rat 255♂. Diet C.		Rat 256♂. Diet 6.	
	Weight.	Food consumed.	Weight.	Food consumed.	Weight.	Food consumed.	Weight.	Food consumed.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
0	41		50		45		43	
4	44	16	56	19	49	18	45	14
8	50	15	64	20	58	21	51	16
12	58	18	64	13	68	25	57	16
16	65	18	59	16	75	29	63	18

As the results of this experiment seemed to warrant further investigations, more studies were started with the purpose of examining the kidneys and non-protein nitrogen of the blood. For the determination of the non-protein nitrogen, blood was obtained by cutting off the tip of the tail which had been pre-

vously immersed in warm water, and allowing the blood to flow into a weighed, oxalated centrifuge tube. From 0.3 to 1.0 gm. samples were thus obtained. The proteins were then precipitated by the addition of 9 parts of a 5 per cent trichloroacetic acid solution. The tube was allowed to stand for $\frac{1}{2}$ hour and then centrifuged. Non-protein nitrogen determinations were made on 2 to 5 cc. samples of the clear, supernatant liquid by the direct Nesslerization method of Folin and Wu (1919). Because of the observations being made upon body weight, food was not with-

TABLE III.

Growth and Non-Protein Nitrogen of Blood of Half Grown Rats on (1) Casein and (2) Casein Plus Cystine Diets.

Days.	Rat 366 ♂.		Rat 367 ♂.		Rat 368 ♂.		Rat 369 ♂.	
	Diet C.				Diet 6.			
	Weight.	Non-protein N	Weight.	Non-protein N.	Weight.	Non-protein N.	Weight.	Non-protein N.
	<i>gm.</i>	<i>mg.</i>	<i>gm.</i>	<i>mg.</i>	<i>gm.</i>	<i>mg.</i>	<i>gm</i>	<i>mg.</i>
0	150		150		155		148	
4	155	50	160	59	173	48	153	37
7	157	45	166	43	178	48	156	47
11	159	42	166	42	182	51	159	46
14	164	44	164	40	188	35	172	34
18	169	41	165	40	186	47	176	45

The kidneys of all these rats appeared entirely normal. The pair of kidneys from each rat had the following weights: Rat 366, 1.43 gm.; Rat 367, 1.38 gm.; Rat 368, 1.46 gm.; Rat 369, 1.49 gm. The respective weights for the normal as given by Donaldson (1924) for rats of the above weights are 1.48, 1.45, 1.61, and 1.53 gm.

held from the animals at any time previous to taking the blood samples. When the rats had developed definite symptoms of nephritis as evidenced by the loss of appetite, decrease in weight, and increase in the non-protein nitrogen of the blood, they were killed and the kidneys removed and weighed. The weights were compared with those of Donaldson (1924), and with the kidney weights of the other rats of the series.

To establish the effect of small amounts of free cystine in the rations of half grown rats, we have subjected four animals of

about 150 gm. of body weight to the same regimen as the younger rats. The data on growth, non-protein nitrogen of the blood, and condition of the kidneys at autopsy as related to the diet are recorded in Table III.

The failure of nephritis to develop in the above series of half grown rats is in accord with the findings of Newburgh, Marsh,

TABLE IV.

Growth, Blood Non-Protein Nitrogen, and Kidney Weights of Rats Receiving Varying Amounts of Cystine.

Rat No.	Diet.	Body weight in gm. after the following days on diet.						Blood non-protein N, mg. per 100 cc. after the following days on diet						Weight in gm. of both kidneys on last day.	
		0	4	8	9	12	16	0	4	8	9	12	16	Found.	Donaldson.
907 ♀	9	43	44	53		64	61	62	52	53		70	109	1 07	0 65
908 ♂	9	41	46	52		47	50	45	47	62		172	90	0 80	0 55
909 ♀	C	44	47	53		57	58	48	55	54		54	48	0 57	0 62
910 ♀	9	43	50	52		54	48	65	54	73		174	294	1 08	0 53
911 ♂	9	43	50	54		54	54	65	55	68		180	146	0 98	0 59
912 ♀	C	44	52	56		60	64	64	56	72		71	50	0 65	0 67
943 ♀	6	42	47	54		60	63	68	46	48		50	151	0 97	0 66
944 ♂	6	47	54	62		60	60	65	44	50		185	159	1.26	0 64
945 ♀	C	44	48	52		58	63	62	46	50		48	62	0.59	0 66
946 ♀	6	46	50	58		66	71	47	48	49		47	79	1 07	0 72
947 ♂	6	42	50	52		50	56	47	50	65		157	97	0 67	0 61
948 ♀	C	38	48	54		58	60	58	50	57		60	54	0 55	0 64
978 ♂	9	45	53	58	55			55			296			1 58	0 59
979 ♀	9	45	53	61	58			53			93			1 23	0 62
980 ♂	6	45	53	56	53			59			166			1 57	0 58
981 ♀	6	42	48	52	49			48			164			1 42	0 54
982 ♀	3	44	52	60	59			50			91			1 06	0 63
983 ♀	3	45	50	58	57			51			97			1 02	0 61
984 ♂	C	45	50	56				45		52				0 61	0 60

Clarkson, and Curtis (1925) and of Addis, MacKay, and MacKay (1926-27). The rats receiving free cystine show an accelerated growth rate as compared to the rats on the diet with no added cystine. This fact is also in accord with the data of Newburgh, Marsh, Clarkson, and Curtis, but is in contrast to the effect noted in the case of the very young rats of the present investigation.

In the following experiments with young rats (see Table IV and compare with Table II) large increases in the non-protein nitrogen of the blood very suddenly occur when, as indicated by the fall in body weight, renal injuries have developed. The same type of swollen kidneys, the loss of appetite, and appearance of drowsiness were encountered as were found in the preliminary experiments. Two additional rations were employed, Diet 3 and Diet 9, containing respectively 0.3 and 0.9 gm. of cystine to replace an equivalent amount of casein in Diet C. The rats were killed on the last day as shown in Table IV and the kidneys immediately removed and weighed. After being split longitudinally, the kidneys were placed for 24 hours in a 10 per cent formaldehyde solution and then prepared for histological examination by the usual procedure of mounting in paraffin. The sections were stained with hematoxylin and eosin. We are indebted to Dr. Hamilton R. Fishback of Northwestern University for the histological data given below in general outline form:

Rat 943.—Extensive necrosis of cortex, most evident in tubules, but involving also numerous glomeruli to complete destruction. Intervening areas show cloudy swelling of tubular epithelium. Necrotic areas are not sharply demarcated. Hemorrhage into necrotic areas is seen. Hyaline casts in the collecting tubules. Diagnosis: acute toxic nephrosis.

Rat 944.—Slight diffuse degeneration of tubular cells, not so extensive as in Rat 943. Patchy deposit of masses of calcium generally showing first in degenerated tubule cells. Diagnosis: acute toxic nephrosis.

Rat 947.—Swelling of tufts with obliteration of capsular spaces. Bloodless glomeruli. Proximal tubules are swollen shut and the lining cells show all changes from cloudy swelling to complete cytolysis, with finely granular debris (necrotic) resulting. Collecting tubules show little change. Blood vessels moderately engorged. No hemorrhage. Diagnosis: acute toxic nephrosis (early toxic changes).

Rat 978.—Adhesion of capsule to surrounding fat with thickening and some mononuclear cell infiltration. Hemorrhage into capsule and in irregular areas in the cortex. Necrosis of whole areas of cortex including glomeruli and tubules. Slight thickening of capsules of undestroyed glomeruli. Numerous hyaline and epithelial cell casts in the straight collecting tubules. Occasional polymorphonuclear cells infiltrating the necrotic areas. Diagnosis: acute toxic nephrosis (slightly later stage than in Rat 943).

Rats 979 and 980.—The sections from Rats 979 and 980 are similar to those from Rat 978.

Rat 981.—Similar in general to Rat 978. Necrosis somewhat more extensive. Hemorrhages marked. Diagnosis: acute toxic nephrosis.

Rat 982.—Similar in general to Rat 943. Slight necrosis of same type as

in Rat 943 but early and much less extensive. Patches are seen which appear normal. Diagnosis: acute toxic nephrosis.

Rat 983.—Similar to Rat 943. Diagnosis: acute toxic nephrosis.

Rats 945 and 984.—Appearance of the tissue is normal. Diagnosis: normal.

The fact that many rats in our earlier work had recovered after restoration to the stock ration, and then after a brief time were returned to the nephrotoxic diet and had had no recurrence of symptoms, suggested that it might be possible to obtain recoveries

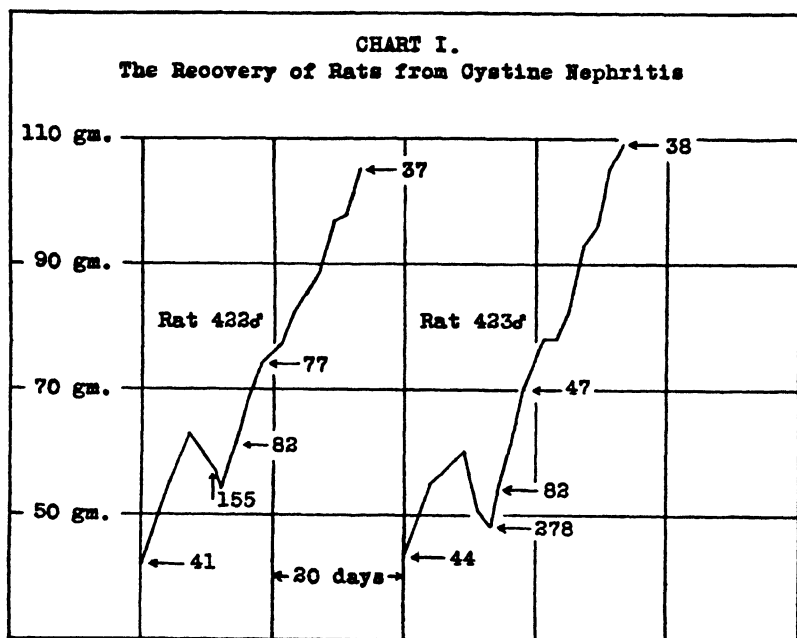


CHART I.

without any change of ration. The fact that some of the rats apparently were not affected by the cystine-containing diet also pointed to this possibility. Two rats were accordingly placed on Diet 6 and their weights, food consumption, and the non-protein nitrogen of the blood followed for 31 days. The growth curves of these rats are shown on Chart I, with the non-protein nitrogen values indicated by arrows at the various stages of growth. The condition of the kidneys at the end of the experiment was as follows:

Rat 422.—No spots, bumpy surface. Weight 1.05 gm.; Donaldson 0.986 gm.

Rat 423.—Covered with white spots. Weight 1.08 gm.; Donaldson 1.016 gm.

DISCUSSION.

The above results indicate that 0.3 to 0.9 per cent of free cystine in the diet is nephrotoxic to young rats of 60 gm. of body weight or less. As rats used for studies in nutrition and metabolism are frequently started before they have reached this weight and since cystine, being an essential amino acid, is often included in synthetic diets, it is apparent that this development is of practical importance and should be recognized and guarded against.

Since the above experiments were completed, Hartwell (1928) has observed renal injuries and death in rats of 40 gm. initial body weight when they were fed rations low in vitamin B and containing 20 per cent of edestin as the sole protein. The kidney injuries found by her are strikingly similar to those observed by us, as shown by the following quotation of her Experiment I.

"Basal diet + 2 g. marmite.

"After about a week several of the rats became weak and were obviously ill, 5 of them died on the 13th day, having shown previous loss in weight. The remaining 7 were killed and *post mortem* examination showed only one to be normal, while the other 6 had kidneys of a deep purple colour and gorged with blood."

The remarkable recovery of our rats without change of ration is duplicated by Hartwell's experiment in which two survivors after 9 weeks had kidneys that "were slightly yellowish but were otherwise normal." No effects were observed by Hartwell with rats of from 85 to 145 gm. body weight. Various samples of highly purified edestin caused the same nephritic symptoms when incorporated in the diets. Casein fed at the same level was harmless. An increase of the vitamin B of the ration from that represented by 2 gm. of marmite to 15 gm. protected the rats entirely from the kidney damage. Marmite autoclaved at 120° for 5 hours was equally effective, indicating that the antineuritic factor is not the active substance. Hartwell says, "It is possible that some specific amino acid present to a greater extent in edestin than in other proteins used for feeding experiments is responsible

for the amount of marmite required." Our findings point to cystine as such a specific amino acid.

SUMMARY.

1. Young rats of 60 gm. of body weight or less develop acute toxic nephrosis when restricted to synthetic diets containing 0.3 to 0.9 per cent of free cystine.

2. Some rats which have developed the symptoms of acute nephrosis, due to dietary cystine, may recover and grow normally without any change in the ration.

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THE COMPOSITION OF SPINACH FAT.

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Spinach contains a fat of great interest. In 1919, it was shown by Osborne and Mendel (1) that spinach is richer in the fat-soluble vitamins than most vegetables. Considerable work has been done since then to differentiate between the growth-producing and antirachitic factors in this fat, thus focusing attention on the unsaponifiable extract of spinach. We have been unable to find in the literature any detailed examination of the fat itself, and, while this may not be of such general interest as the vitamin-bearing fraction, nevertheless it is valuable in that it gives us more complete knowledge of the occurrence of these biologically active materials, knowledge that may assume increasing significance as the study of vitamins progresses.

EXPERIMENTAL.

68 kilos of dried spinach were exhaustively extracted with cold acetone, and the extract obtained by concentration under nitrogen at reduced pressure. This was dissolved in 8 liters of boiling alcohol, allowed to stand overnight, and the insoluble material which separated was filtered off. Report of the examination of this material will be made in connection with the unsaponifiable fraction.

The alcohol was then removed by distillation, the residue dissolved in ether, and washed with 1 per cent H_2SO_4 . This washed ether solution was then extracted with 10 per cent KOH to remove free fatty acids. The KOH extract was acidified, extracted with ether, and the free fatty acids, mixed with considerable chlorophyll, obtained as a green, sticky mass upon evaporation of the ether. Yield 400 gm. This was distilled at 2.5 mm. to remove chlorophyll and its degradation products. Yield 290 gm. of a brown oil,

mostly distilling at 190°. A few gm. of tarry substance caught in the ice-cooled trap of the still had an odor suggesting volatile acids, but when steam-distilled no acids could be recovered as the silver salts.

Free Fatty Acids.—This fraction was redistilled at 2.5 mm. and a small amount of solid filtered off from the distillate. Recrystallized from alcohol, this melted at 57–58°. The material contained in the filtrate was added to the main portion of the liquid distillate, which was dissolved in ether and extracted first with K_2CO_3 , and then with KOH. The KOH extract weighed 9.1 gm. and was put aside for later work.

The K_2CO_3 extract was separated by the Gusserow-Varrentrapp method ((2) p. 556) into 53 gm. of liquid acids and 10 gm. of solid acids.

These solids were joined to those filtered from the liquid distillate above, and crystallized several times from hot alcohol, until a melting point of 62.3–62.5° (corrected) was obtained. This was unchanged by further crystallization.

0.1215 gm. substance: 0.3331 gm. CO_2 , 0.1377 gm. H_2O .

$C_{16}H_{32}O_2$.	Calculated.	C 74.9,	H 12.6,	neutralization value 219.
	Found.	" 74.8,	" 12.7,	" " 222.

The solid acids were thus, chiefly at least, palmitic acid.

The liquid acids had an iodine number of 181. This fraction was added to a similar fraction obtained from the neutral fat for further examination.

Fatty Acids of the Neutral Fat.—The ether solution from which the free fatty acids had been extracted with 10 per cent KOH solution was concentrated and saponified with alcoholic potash. The soaps were extracted with ether to remove the unsaponifiable fraction, and the fatty acids obtained by acidifying the soaps and extracting the now free acids with ether. 260 gm. of crude fatty acids were thus obtained. These were distilled at 2 mm. pressure, the distillate dissolved in ether, and successively extracted with $(NH_4)_2CO_3$, K_2CO_3 , and KOH solutions. Steam distillation of the $(NH_4)_2CO_3$ extract yielded volatile acids equivalent to but 4.5 cc. of normal base. The residue of the $(NH_4)_2CO_3$ extract, the KOH extract, and the residue left in the ether after the KOH extract had been made, were all negligible and were discarded.

The acids of the K_2CO_3 extract, when dissolved in a small amount of alcohol, crystallized out 12.5 gm. of solids. The filtrate from this solid was separated by the Gusserow-Varrentrapp method into 180 gm. of liquid acids with an iodine number of 137, and 4 gm. of solid acids.

The solid portions were all joined, and fractionally crystallized from alcohol.

TABLE I.
Saturated Fatty Acids.

Fraction No.	Weight.	M.p.	Neutralization value.	C	H
	<i>gm.</i>	<i>°C.</i>		<i>per cent</i>	<i>per cent</i>
1	0.8	75.5–76	147	78.25	13.11
2	2.0	52–54	204	75.29	12.61
3	4.8	54–56	211		
4	2.5	57–58.5		74.94	12.61
Calculated $C_{16}H_{32}O_2$.		62.5	219	74.91	12.61
“ $C_{18}H_{36}O_2$.		69	197.5	75.95	12.78
“ $C_{20}H_{42}O_2$.		77	142	78.69	13.24

TABLE II.
Distillation of Liquid Fatty Acids.

Fraction No.	Weight.	Temperature.	Pressure.	Neutralization value.	Iodine No.
	<i>gm.</i>	<i>°C.</i>	<i>mm.</i>		
1	6.0	To 170	0.4	181	117
2	18.6	170–180	0.35	186	155
3	44.3	180–190	0.25	200	144
4	54.6	190–200	0.35	200	133
5	21.5	200–207	0.35	198	127

The data in Table I indicate that Fraction 1 is cerotic acid, contaminated with, perhaps, arachidic acid. Fractions 2 and 3 are mixtures of palmitic and stearic acids, and Fraction 4 is practically pure palmitic acid.

Liquid Acids.—The liquid fractions from the free fatty acids and from the neutral fat were now joined and fractionally distilled three times, with the results shown in Table II.

These fractions were then brominated by dropping free bromine

into a 10 per cent solution of the acid in ether, the temperature being kept below 0° . After standing several hours, the insoluble portion was filtered and washed with cold ether, dried, and weighed. The melting point of each fraction was the same, $180-181^{\circ}$, with no blackening. The weights are given in Table III. Fraction 4 was recrystallized from benzene, and bromine determined.

0.1774 gm. substance: 0.2646 gm. AgBr.

$C_{18}H_{30}O_2Br_6$. Calculated. Br 63.32.

Found. " 63.45.

This checks for linolenic hexabromide, indicating the presence of linolenic acid in the fatty acids.

TABLE III.
Bromination of Unsaturated Acids.

Fraction No.	Weight taken.	Hexabromides.	Solid tetrabromides.	Oily tetrabromides.	Dibromides.	Unsaturated ester from dibromides.	Iodine No., unsaturated ester.
	gm.	gm.	gm.	gm.	gm.	gm.	
1	2.5	0.5	None.	None.	3.6	1.8	115
2	2.4	0.7	"	"			
3	20	7.3	3.1	19.3	4.3	2.1	111
4	15	6.1	6.2	9.7	6.2	3.4	124
5	10	2.1	0.5	None.	4.4	2.2	115

The ethereal solution, after separation of the insoluble material, was washed with $Na_2S_2O_3$, dried, the ether removed, and the residue taken up in hot petroleum ether. On cooling, a yellow, sticky mass separated which on long standing further separated into a light yellow solid and a brown oil. The solid was recrystallized from petroleum ether and the last traces of color removed with animal charcoal. The two highest melting samples were analyzed for bromine, as follows:

Fraction 3. 0.1259 gm. substance: 0.1595 gm. AgBr.

" 4. 0.1152 " " : 0.1458 " "

$C_{18}H_{32}O_2Br_4$. Calculated. Br 53.33, m.p. $113-114^{\circ}$.

Fraction 3. Found. " 53.90, " $110-111^{\circ}$.

" 4. " " 53.85, " $112-113^{\circ}$.

This checks for linoleic tetrabromide, indicating linoleic acid among the fatty acids.

The brown oil in Fraction 3 was separated from the petroleum ether mother liquor, and its bromine removed by the method of Wesson (3). The resulting unsaturated ester had an iodine number of 148. This was saponified and oxidized with dilute alkaline permanganate ((2) p. 575). A small amount of material melting at 164–166°, and having the solubilities of sativic acid, was obtained.

0.1418 gm. substance: 0.3174 gm. CO₂, 0.1298 gm. H₂O.

C₁₈H₃₂O₂(OH)₄. Calculated. C 62.0, H 10.4.

Found. " 61.1, " 10.3.

These data suggest the material is impure sativic acid, and that therefore the oily, petroleum ether-insoluble bromides are apparently an isomeric form of linoleic tetrabromide.

The clear yellow petroleum ether solutions were evaporated to dryness after the separation of the tetrabromide fraction, and the residues weighed. These were taken up in a small amount of fresh petroleum ether, filtered to remove as much tetrabromide as possible, taken to dryness again, and the bromine removed. The resulting esters were saponified and oxidized with alkaline permanganate, yielding a small amount of a white powder melting at 128–129°, and having the solubilities of dihydroxystearic acid.

0.1292 gm. substance: 0.3222 gm. CO₂.

C₁₈H₃₄O₂(OH)₂. Calculated. C 68.2. Found. C 3.0.

These data check for dihydroxystearic acid, proving the presence of oleic acid in the original mixture of fatty acids.

The weights of the various bromide fractions are collected in Table III.

When these weights are calculated to the basis of the whole fraction as recorded in Table II, it will be seen that from the total of 145 gm., there were recovered the equivalent of 18.5 gm. of linolenic acid, 50.3 gm. of linoleic acid, and 38.1 gm. of oleic acid. There was no evidence of arachidonic acid.

SUMMARY.

68 kilos of spinach yielded 550 gm. of fatty acids, of which 47 per cent were present as glyceride and 53 per cent free. From

the total fatty acids, 26.5 gm. of solid acid were isolated, though the means of separation was by no means quantitative. These solid acids were shown to consist chiefly of palmitic and stearic acids, with about 3 per cent cerotic acid. 145 gm. of liquid acids were recovered from the fractionation processes, and this amount was shown to contain at least 12.7 per cent linolenic acid, 34.7 per cent linoleic acid, and 26.3 per cent oleic acid. Volatile fatty acids were present in traces, if at all.

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THE UNSAPONIFIABLE FRACTION FROM SPINACH FAT.

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(Received for publication, February 13, 1929.)

It is well known that the vitamin activity of a fat resides in its unsaponifiable fraction. Osborne and Mendel (1) have shown that this fraction of spinach fat restores growth to rats deprived of the growth-promoting factor (now known as vitamin A) when added in quantities of 66 mg. per rat per day.

Goldblatt and Zilva (2) agreed that while spinach had the growth-promoting factor, it failed to have the antirachitic factor (vitamin D). There has been a great deal of discussion, particularly by the English workers, as to whether or not spinach contains any appreciable amount of vitamin D, and whether irradiation successfully renders spinach antirachitic. Recently Roscoe (3) has shown the presence of a small antirachitic effect. In the meantime, Japanese investigators (4) claim to have isolated from the unsaponifiable fraction of spinach pure vitamin A, which they have named biosterin. This is a liquid boiling at 147° at 0.02 mm., and whose analysis agrees with the formula $C_{27}H_{46}O_2$ (C 81.0 per cent, H 11.0 per cent). A discussion of this work has been presented by Drummond, Channon, and Coward (5).

In this paper, we have attacked the problem from a chemical, rather than biological, standpoint, and have attempted to isolate such individual compounds as we could, in an endeavor to form a more complete chemical background for the biological investigations.

EXPERIMENTAL.

The unsaponifiable fraction of spinach fat as we obtained it from the acetone extract of spinach (6) consisted of two main portions, (a) the alcohol-insoluble material which was separated by filtration, and (b) the material which was extracted by ether from the alkaline soap solution.

Isolation of a Hydrocarbon.—The alcohol-insoluble material (a) was a dark green, gummy mass. It was boiled with 10 per cent alcoholic potash, diluted with water, and the aqueous alkaline solution extracted with ether. The ether was removed and the residue crystallized from alcohol. 20 gm. of small plates, melting at 67.5–68.5°, separated. The melting point was unchanged by further crystallization from alcohol or from acetic anhydride.

0.1100 gm. substance: 0.3421 gm. CO₂, 0.1415 gm. H₂O.

C₂₀H₄₂. Calculated. C 85.0, H 15.0.

Found. " 84.8, " 15.0.

2.192 mg. substance: 20.478 mg. camphor, Δ 15°.

C₂₀H₄₂. Calculated. Mol. wt.* 282. Found. 285.

* Rast, K., *Ber. chem. Ges.*, **55**, 1051 (1922).

The substance is a hydrocarbon, and apparently isomeric with the eicosane (C₂₀H₄₂, m. p. 38°) reported by Levene, West, and van der Scheer (7). It may be identical with the petrosilane or laurane (both C₂₀H₄₂, m. p. 69°) listed by Lewkowitsch (8).

The aqueous alkaline solution from which the hydrocarbon had been extracted with ether contained nothing but dark amorphous material, probably degradation products of chlorophyll.

Isolation of the Sterol.—The ether extract of the aqueous soap solution (b), constituting the other main portion of the unsaponifiable fraction, and containing 119 gm. of material, was taken to dryness and crystallized from 95 per cent alcohol to separate the crystalline material. The filtrate containing the non-crystalline fraction will be reported later in this paper.

The solids were systematically fractionally crystallized from alcohol. Seven fractions were obtained, as shown in Table I.

These fractions were examined further as follows:

Fraction 1 consisted of approximately 90 per cent of cholesterol-like plates, which further crystallization from alcohol failed to improve. Crystallized once from ether, it gave prisms that melted at 162–165°. The mother liquor from the first ether crystallization was allowed to concentrate, and a second crop, melting at 158–163°, separated. The yield of this material was 2.1 gm.

Fraction 2, by crystallization at 60° from alcohol, gave 1.25 gm. of plates melting at 155–163°, but the filtrate then set to a

solid gelatinous mass on cooling and we were not successful in isolating more of this individual substance by this means.

Fractions 3 and 4, by a hot crystallization from alcohol, yielded a further quantity (0.65 gm.) of plates that melted at 167° very sharply. Further fractional recrystallization from alcohol of the material in the filtrate accomplished absolutely nothing.

Fractions 5 to 7 were also crystallized from hot alcohol and yielded 1.9 gm. of sterol melting at 156–158°. Altogether, approximately 10 gm. of this sterol were isolated. This will be carefully characterized in a later paper. The following determinations were made on a sample melting at 165–167° after repeated crystallization.

0.3054 gm. substance in 20 cc. of CHCl_3 in a 2 dm. tube rotated + 0.15° (saccharimeter scale).

$$[\alpha]_D^{25} = \frac{+0.15^\circ \times 20 \times 0.347}{2 \times 0.3054} = +1.7^\circ.$$

0.1107 gm. substance: 0.0046 gm. loss *in vacuo* at 100°, 0.3238 gm. CO_2 , 0.1117 gm. H_2O .

$\text{C}_{27}\text{H}_{46}\text{O} \cdot \text{H}_2\text{O}$. Calculated. H_2O 4.45, C 80.1, H 11.5.

Found. " 4.16, " 79.8, " 11.3.

TABLE I.

Fractional Crystallization of Solid Unsaponifiable Fraction.

Fraction No.	Weight.	Melting point.
	gm.	°C.
1	4.6	154–161
2	4.5	80–117
3	5.1	80–130
4	3.4	75–130
5	1.0	70–124
6	2.6	70–133
7	2.9	

The material gave a strong blue coloration with the Liebermann-Burchard test; the Salkowski reaction was positive and similar to that of ergosterol.

Aliphatic Alcohols.—The mother liquors from Fractions 2 to 4 were taken to dryness and the residue crystallized from ether in rather dilute solution at -10° . About 3 gm. of material melting sharply at 76–77° were isolated.

4.813 mg. substance: 14.273 mg. CO_2 , 6.126 mg. H_2O .

$\text{C}_{22}\text{H}_{46}\text{O}$. Calculated. C 80.9, H 14.2.

Found. " 80.8, " 14.2.

2.814 mg. substance: 34.10 mg. camphor, Δ 10.5°.

$\text{C}_{22}\text{H}_{46}\text{O}$. Calculated. Mol. wt. 326. Found. 315.

The Liebermann-Burchard test was negative.

Further recrystallization did not change the melting point or per cent composition. The substance is apparently an isomer of the docosyl alcohol ($\text{C}_{22}\text{H}_{46}\text{O}$, m. p. 73–74°) reported by Levene, West, and van der Scheer (7).

The mother liquors from Fractions 5 to 7 were taken to dryness, and the residue separated by crystallization from methyl alcohol into two fractions melting at (a) 68–70° and (b) 67–69°. The fraction (a) yielded a further quantity of the docosyl alcohol upon crystallization from ether; fraction (b) yielded as a top fraction, after repeated crystallization, 0.2 gm. of a material melting at 87–88°.

3.985 mg. substance: 11.356 mg. CO_2 , 4.879 mg. H_2O .

$\text{C}_{24}\text{H}_{50}\text{O}_2$. Calculated. C 77.8, H 13.6.

Found. " 77.7, " 13.7.

2.595 mg. substance: 21.442 mg. camphor, Δ 13.0°.

$\text{C}_{24}\text{H}_{50}\text{O}_2$. Calculated. Mol. wt. 270. Found. 272.

The Liebermann-Burchard test was negative.

We have been unable to find mention of any similar material in the literature. We believe it to be a dihydric aliphatic alcohol. It, as well as the isomer of docosyl alcohol, will be more completely characterized in a later paper.

The mother liquors from all the separations in this section were joined and small amounts of the sterol and of the docosyl alcohol could with difficulty be separated. We have no reason to believe there is any other chemical individual in this fraction.

Filtrate from Solid Fraction.—After as much of the solid material as possible had been separated from the original unsaponifiable fraction by crystallization from 95 per cent alcohol, and a number of intermediate indeterminate semisolid fractions discarded, the residue was dissolved in ether and made up to a volume of 300 cc. It contained about 75 gm. of material. It was deep red in color, but on further examination it was found that the xanthophyll exceeded the carotin present.

The sterol had been removed almost completely. 0.6483 gm. was dissolved in 50 cc. of alcohol and precipitated with digitonin by the Windaus method. The digitonide weighed 0.1350 gm., indicating that 5.2 per cent of the oil was sterol.

A portion of the oil freed from sterol by digitonin precipitation showed an iodine number of 137.

The oil was fractionally distilled, with the results shown in Table II.

Of these fractions, Fraction 2 was a freely distillable, not viscous oil with some suggestion of purity. The high boiling fraction was secured only with decomposition, the high vacuum being lost three times and the heating stopped until it was recovered, so that the distillate is a viscous decomposition product.

Fraction 2.

2.099 mg. substance: 6.336 mg. CO_2 , 2.586 mg. H_2O .

$\text{C}_{27}\text{H}_{44}\text{O}$. Calculated. C 82.2, H 13.8.

Found. " 82.3, " 13.8.

TABLE II.
Distillation of Liquid Unsaponifiable Fraction.

Fraction No.	Temperature of bath.	Temperature of distillate.	Pressure.	Weight. *
	°C.	°C.	mm.	gm.
1	189-220	110-172	0.9-0.85	2.1
2	220-249	172-188	0.85	5.0
3	249-285	188-200	0.85-1.2	1.9
4	350	280	2.0	8.1

The oil was dissolved in glacial acetic acid and brominated with a slight excess of bromine in the same solvent. Nothing crystalline could be separated. The reaction mixture was treated with cold water, and the oily bromide was extracted with ether. The ether solution was thoroughly washed, dried, and taken to dryness in a vacuum desiccator over paraffin, KOH, and CaCl_2 . The oil was analyzed.

9.844 mg. substance: 14.613 mg. AgBr.

$\text{C}_{27}\text{H}_{44}\text{OBr}_2$. Calculated. Br 28.8. Found. 28.8.

3.60 mg. substance: 46.45 mg. camphor, Δ 5.5°.

$\text{C}_{27}\text{H}_{44}\text{OBr}_2$. Calculated. Mol. wt. 554. Found. 563.

It therefore appears that this oil belongs to the open chain series rather than to the ring compounds of the sterol class. The fact that it has 27 carbon atoms, a double bond, and 1 oxygen atom, strongly suggests that it is either formed from, or is a predecessor of the sterol in the plant tissues.

In conclusion, we wish to express our thanks to Mr. Harold Emerson for the micro analyses reported above.

SUMMARY.

From the unsaponifiable fraction of spinach fat, there has been isolated a hydrocarbon of the formula $C_{20}H_{42}$, a phytosterol of the usual formula $C_{27}H_{46}O$, melting at $165-167^{\circ}$, two alcohols, $C_{22}H_{46}O$ and $C_{24}H_{50}O_2$, and an unsaturated, oily, compound having the formula $C_{27}H_{54}O$.

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THE RÔLE OF THE PHOSPHOLIPIDS OF THE INTESTINAL MUCOSA IN FAT ABSORPTION.

WITH ADDITIONAL DATA ON THE PHOSPHOLIPIDS OF THE LIVER, AND SMOOTH AND SKELETAL MUSCLE.*

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INTRODUCTION.

It is almost universally believed that neutral fat must undergo saponification in the lumen of the small intestine before it is absorbed. It has been known since the time of Munk (1880) that the lipid of the thoracic lymph, which acts as the chief vehicle for the transportation of absorbed fat into the blood, consists mainly of neutral fat. Furthermore, the analysis of the chyle from the mesenteric lymphatics has shown that 95 to 97 per cent of the fatty acids is in the form of the triglycerides (Moore, 1903). In 1842 Goodsir discovered that there is an accumulation of fat-like droplets within the epithelial cells of the intestinal mucosa during fat absorption, and some years later Perewoznikoff (1876) observed that the same phenomenon occurs after feeding fatty acids and glycerol, but it was not until chemical methods were applied (Moore, 1903; Noll, 1910) that it was proved that this accumulated material consists mainly of neutral fat. These facts led to the conclusion that there is a resynthesis of neutral fat from its absorbed constituents within the intestinal mucosa, presumably in the epithelial cells.

It has been tacitly assumed that this resynthesis is simply a reversal of the hydrolysis which takes place in the lumen of the intestine; that is, it is enzymic in nature. Nevertheless, attempts to demonstrate *in vitro* the synthesis of fat from its split products in the presence of hashed mucosa have been either inconclusive or definitely unsuccessful. Ewald (1883) and Hamburger (1900) believed that their experiments proved that such a synthesis did occur *in vitro* but their technique has been severely criticized by Moore (1903) whose own experiments gave negative results. Likewise

* Part of the material presented in this paper is taken from a thesis submitted by the author to the faculty of the University of Rochester in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Frank and Ritter (1906) were unable to detect any synthesis of neutral fat when soap and glycerol were incubated with hashed mucosa.

With the old theory in mind (Krehl, 1890) that the mitochondria of the epithelial cells were the active agents in fat synthesis, and the more recent evidence (Mayer, Rathery, and Schaeffer, 1914) that mitochondria consist of phospholipid, it was thought that the phospholipids of the epithelium might be an intermediary stage in the resynthesis of fat from its absorbed constituents. If this be true, the phospholipids of the mucosa, and more particularly their constituent fatty acids, should assume the characteristics of the fat which is being absorbed. This idea was the basis for the following investigation.

EXPERIMENTAL.

The general plan of the experimentation was to feed a fat of pronounced characteristics and to observe the effects on the amount and nature of the phospholipid of the intestinal mucosa. The degree of unsaturation, as measured by the iodine number (I.N.), was chosen as the most convenient characteristic for studying changes in the composition of the phospholipids and their fatty acids. The experiments were conducted in pairs—one control and one fat absorption experiment—in order to nullify such errors in the I.N. as might be caused by oxidation or slight differences in technique.

The experimental procedure was as follows: Two cats were fed on a constant diet for at least 4 days. On the day of the experiment, either cod liver oil (I.N. 165) or cocoanut oil (I.N. 10), in the form of a 50 per cent emulsion, was administered by stomach tube to one of these cats; some hours later, the animal was killed. The other cat was killed in a postabsorptive condition to serve as a control. The mucosa and muscle of the small intestine were extracted separately with hot alcohol. The phospholipids were separated and purified by acetone precipitation from ether solution and, after saponification, the weight and I.N. of the constituent fatty acids were determined.

Preparation of Material.—The cats were anesthetized with illuminating gas and then bled to death.

The mucosa and muscle of the small intestine were separated in the following manner: About 20 to 25 cm. from the end of the

ileum the intestine was firmly clamped. Then, with the shorter end held taut, the point of a moderately fine pair of scissors was inserted between the muscle and the mucosa. By carefully sliding the scissors forward the circular fibers were severed and then, with moderate traction, the two parts could easily be separated.¹ The piece of mucosa was slit open, rinsed in two lots of warm 50 per cent alcohol, and then immersed in 95 per cent alcohol. The remainder of the intestine was readjusted in the clamp and the process repeated. As soon as the separation had been completed the moist weights of the mucosa and the muscle were determined. The moist weight of the mucosa was found by weighing the beaker of alcohol before and after the tissue had been added. This weight cannot be considered as more than a fair approximation because an indefinite amount of the rinsing alcohol undoubtedly adhered to the mucosa. The average weight of the mucosa was 67 gm. when moist and 10.5 gm. when dry; for the muscle the average weights were 38 and 7.6 gm. respectively. The muscle was hashed in a meat grinder, but it was found more convenient to use scissors for hashing the mucosa.

In the last series of experiments, while the author was preparing the small intestine for extraction, an assistant prepared the liver and skeletal muscle. First, 40 gm. of the hashed liver were weighed out and covered with alcohol. Then the muscles of the right hind leg of the cat were stripped from the bones and passed twice through a meat grinder. After being mixed well, 40 gm. were weighed out in a bottle and covered with alcohol. In these experiments the extraction of the mucosa and liver was begun immediately; the intestinal and skeletal muscles were covered with alcohol and kept in the refrigerator until it was possible to extract them.

The method used for the extraction and separation of the lipids, substantially that developed by Bloor (1926), was as follows:

Extraction.—The hashed tissue was extracted with hot alcohol in the apparatus used in this laboratory (Sperry, 1926). After extraction for 2 hours the tissue was dried and weighed. Then it was pulverized in a mortar and extracted for a 3rd hour. The

¹ Histological sections were made of both the mucosa and the muscle and it was found that the latter was practically free from adherent submucosa.

completeness of the extraction process was determined by digesting about 40 gm. of the tissue residue in strong alkali, acidifying, and extracting with ethyl ether. The ether extract was distilled to dryness. The lipids were extracted from the ether residue with petroleum ether, dried, and weighed. Calculated for 100 gm. of dried tissue, the amounts of unextracted lipids were: in the mucosa 0.001 gm., in the intestinal muscle 0.018 gm., in the skeletal muscle 0.005 gm., and in liver 0.065 gm.

Separation of the Phospholipids.—The combined alcoholic extracts were distilled on the steam bath *in vacuo*; when the distillation was nearly complete, the temperature was not allowed to rise above 40°. The ethyl ether solution of the lipids was distilled to a small volume and then rinsed into 4 volumes of acetone. After centrifugalization, the acetone solution was poured off, the precipitate was well scrubbed with fresh acetone and again centrifuged.

In the first part of the investigation only the ether-soluble portion of the acetone-insoluble material was employed, since it was believed that this constituted the real phospholipid. The ether-insoluble material was discarded. The phospholipids were reprecipitated with acetone from the clear ether solution, centrifuged, and redissolved in 25 cc. of anhydrous ether; then 5 cc. aliquots were taken for weight and I.N. determinations, and the remainder was saponified.

In the latter part of the investigation, the procedure was modified in several respects. In order to secure more complete precipitation of the phospholipid, 2 cc. of a saturated alcoholic solution of magnesium chloride were added to the acetone every time the phospholipid was precipitated or scrubbed. Furthermore, it was decided to omit the determination of the weight and I.N. of the phospholipid, since the results up to this time had clearly shown that the data obtained were much less significant than the data on the constituent fatty acids. Also, from then on, the whole of the acetone-insoluble material was saponified, as it was no longer necessary to discard the portion which was insoluble in ether.

Saponification and Extraction of the Fatty Acids.—The phospholipid was saponified on the steam bath with 2 to 4 gm. of stick sodium hydroxide and 100 cc. of 50 per cent alcohol. After boiling

for 3 to 4 hours, the solution was acidified to Congo red with concentrated hydrochloric acid and 25 cc. of water were added in order to reduce the alcoholic concentration to 40 per cent. The fatty acids were extracted completely with petroleum ether. If the fatty acids were present in sufficient amount, the petroleum ether solution was adjusted to a volume of 25 cc., and aliquots were taken for weight and I.N. determination; otherwise, the total amount was dried and weighed.

Drying.—The samples of the phospholipid and the fatty acids were freed from solvent on the steam bath, with a stream of CO_2 to prevent oxidation. The samples which had been taken for I.N. determination were removed as soon as the solvent had evaporated, but those to be weighed were heated for 15 minutes and then kept in a vacuum desiccator for $\frac{1}{2}$ to 1 hour.

Determination of Iodine Number.—The Hanus method was used throughout. In every experiment the I.N. of the weighed sample was determined, and these values have been given in Tables I to V. It is evident that the fatty acids are oxidized to some extent during the drying process, since the I.N.'s of the undried samples range from 0 to 5.7 per cent and average 1.9 per cent higher than those of the corresponding weighed samples.

DISCUSSION.

Series I. Effect of Cocoonut Oil and Cod Liver Oil on the Phospholipids of the Intestinal Mucosa.

The original plan of the investigation was to find out whether the phospholipid fatty acids from the wall of the small intestine of a cat absorbing cocoonut oil have a lower I.N. than those of a control cat. The results of the few experiments carried out on this plan were inconsistent. It seemed likely that the phospholipid of the muscle, which comprises a considerable proportion of the wall of the intestine, would tend to mask any change which might take place in the composition of the phospholipid of the mucosa. Furthermore, it was realized that, if absorbed fat does produce a change in the I.N. of the phospholipids of the absorbing mucosa, this change should be demonstrated most effectively when two fats of widely different I.N. are fed.

Accordingly, in the following experiments only the phospholi-

TABLE I.

Influence of Fat Absorption on Phospholipid of Mucosa of Small Intestine.

Experimental conditions.	Cat No.*	Phospholipid.		Fatty acids of phospholipid.	
		Weight in dry mucosa.	Iodine No.	Per cent.	Iodine No.
Control cats, postabsorptive.	13 c	<i>per cent</i> 5.37	70	70	89
	15 d	10.21		55	89
	17 e	11.33	53	46	92
	19 f	11.33	62	51	99
	21 g	10.29	61	49	95
	21 MM	12.45	61	54	95
	23 "	10.63	58	53	94
	25 "	11.64	53	49	90
Average.....		10.41	60	53	93±1.2
Fed 30 to 40 gm. cod liver oil 6 hrs. before death.	8 a	6.89	56	43	102
	10 b	11.51		52	109
	12 c	7.10	70	55	105
	14 d	9.71		54	103
	16 e		56	44	104
	22 MM	11.63	64	51	104
	24 "	9.12	62	47	103
	26 "	10.74	67	53	108
Average.....		9.53	63	50	106±1.0
Fed 28 to 40 gm. coconut oil 6 hrs. before death.	5	5.17	60	64	81
	9 a	6.80	52	51	85
	11 b	5.05	71	64	89
	18 f	12.03	55	48	93
	20 g	8.21	60	50	97
Average.....		7.45	60	55	89±2.8

* Paired experiments are designated by the same letter. Cats 5 to 11 were kept for 4 days on diet of fat-free beef organs flavored with Liebig's extract; this was poorly eaten. Cats 12 to 21 were fed potatoes and meat extract for 4 days before use; Cats 21 to 26 MM were fed potatoes and white of egg.

pid of the mucosa was studied, and the I.N.'s of the phospholipid fatty acid were compared during the absorption of cocoanut oil and cod liver oil. The results set forth in Table I show decisively that the fatty acids of the ingested fat are incorporated into phospholipid within the absorbing mucosa. Thus, in the control cats the average I.N. of the phospholipid fatty acids is 93 ± 1.2 , while in the cats absorbing cod liver oil the I.N. is 106 ± 1.0 ; with cocoanut oil the results are less consistent, but, in general, they indicate a lower I.N. than in the controls.

It may be seen in Table I that the average percentage of fatty acids in the phospholipid of the mucosa is about 53, a value which is much lower than the theoretical for pure phospholipid (about 72). This discrepancy is probably explained by the presence of "carnithin," an ether-soluble, acetone-insoluble substance (MacLean and MacLean, 1927), in the so called phospholipid. It may be noticed too that the values for the amount of phospholipid in the mucosa show a wide range of variation, although it is apparent that the agreement between corresponding experiments is fairly good. Undoubtedly, the low values in the earlier experiments are chiefly due to imperfect extraction of the phospholipids.

From the I.N.'s it can be calculated that 16 per cent of the phospholipid fatty acids of the absorbing mucosa consist of the fatty acids of cod liver oil. But, before it may be concluded that this extensive transformation of absorbed fatty acids into phospholipid is a step in their resynthesis into neutral fat, the following propositions must be considered: (1) According to Bloor (1916) the increase in phospholipid in the blood during fat absorption is due to synthesis from neutral fat within the red blood corpuscles. Is it not possible that the observed transformation of absorbed fatty acids into phospholipid within the intestinal mucosa is the result of the presence of blood in the tissue? (2) Is it not possible that within the intestinal mucosa phospholipid is synthesized, and absorbed into the blood stream? (3) Cannot the observed phenomenon be an indication that phospholipid is undergoing a continuous catabolism and resynthesis? If this be true, it is likely that the absorbed fatty acids are utilized in the replacement of the phospholipid which has been burned.

It is believed that the marked uniformity of the results, the absence of any increase in the percentage of fatty acids in the

phospholipid, and the magnitude of the change are satisfactory evidence that the observed change in I.N. is not due to contamination of the phospholipid by the ingested fat or fatty acids.

Series II. Effect of Coconut Oil on the Phospholipid Fatty Acids of the Mucosa and Muscle of the Same Intestine.

Obviously, the next step in the investigation was to find out if, during the absorption of fat, the phospholipid of a non-absorb-

TABLE II.
Fatty Acids of Phospholipid from Small Intestine.
Preliminary diet of beef kidney was fed for 2 weeks.

Experimental conditions.	Cat No.	Mucosa.			Muscle.		
		Weight in:		Iodine No.	Weight in:		Iodine No.
		Moist mu- cosa.	Dry mu- cosa.		Moist mus- cle.	Dry mus- cle.	
		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
Control cats; killed 18 hrs. after last feed- ing.	1	1.13	6.02	106	0.43	1.79	109
	2	1.00	7.04	108	0.37	1.83	103
	7	1.08	6.96	114	0.54	3.06	108
	9	1.02	6.36	114	0.52	2.66	113
	11	0.98	5.82	118	0.33	1.57	117
	12	1.02	5.83	111	0.34	1.59	114
	13	0.97	6.44	110	0.49	2.32	94
Average.....		1.03	6.35	112±1.6	0.43	2.12	108±2.9
Fed 28 to 30 gm. coconut oil 6 hrs. before death.	3	0.91	5.68	92	0.42	1.97	91
	5	0.64	3.79	101	Some lost.		98
	8	1.06	6.12		0.40	1.79	106
	10	1.00	6.07	108	0.37	1.67	116
	14	0.97	6.44	110	0.35	1.79	111
Average.....		0.92	5.60	103±4.1	0.39	1.81	104±4.5

ing tissue undergoes a change in composition comparable with that observed in the intestinal mucosa. The intestinal muscle was chosen as a convenient and very suitable tissue for this purpose. In order to establish a constancy in the phospholipids of the intestine, a number of cats were fed on an exclusive diet of raw, lean beef kidney. It was not realized that the feeding of the

highly unsaturated fatty acids of the kidney lipids would influence the degree of unsaturation of the phospholipids. Consequently, when it was found that the phospholipid fatty acids of the mucosæ of the postabsorptive control cats had a higher I.N.

TABLE III.
Fatty Acids of Acetone-Insoluble Lipids from Carcass of Rat.

	Control rats, postabsorptive.			Fed 2 gm. olive oil 4 and 8 hrs. before death.		
	Weight in:		Iodine No.	Weight in:		Iodine No.
	Moist tissue.	Dry tissue.		Moist tissue.	Dry tissue.	
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
Series E.	0 758	3 03	128	0 745	2 97	127
	0 817	3 15	126	0 746	2 91	126
	0 751	2 95	130	0 745	3 02	133
Average.....	0 775	3 04	128	0 745	2 97	129
Series S.				Fed olive oil for from 6 hrs to 4 days before death.		
	0 675	2 80	135	0 736	2 88	135
	0 735	3 00	134	0 733	2 98	133
				0 711	2 98	133
				0 714	2 99	133
				0 786	3 14	129
Average... ..	0 705	2 90	135	0 736	2 99	133
General average...	0.747	2.99	131±1.7	0.740	2.98	131±1.2

Rats of Series E were fed for more than 2 weeks on a diet of 25 per cent alcohol-extracted casein, 71 per cent corn-starch, and 4 per cent Osborne and Mendel² salt mixture, with daily addition of a small amount of yeast for vitamin requirements. They were exercised for 8 hours before death.

Rats of Series S were fasted for 5 days and then fed for 7 days on the diet mentioned above.

than had been obtained in the earlier experiments with cod liver oil, it was thought wise to use cocoanut oil, in spite of its previous unsatisfactory performance.

Only the data on the phospholipid fatty acids are given in Table II. Despite lower values for the average in the absorbing cats

² Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

than in the controls, it is apparent that there is no significant change in the amount of phospholipid fatty acids in either the mucosa or the muscle during the absorption of fat. With respect to the I.N.'s of the phospholipid fatty acids, the results are very inconsistent. In the mucosæ of the postabsorptive controls the average I.N. is 112 ± 1.6 , while in the mucosæ of the cats which were absorbing cocoanut oil the I.N. is 103 ± 4.1 , results which agree with the previous observations in showing that the absorbed fatty acids are transformed into phospholipid. However, the primary purpose of this series of experiments was to see if there is a change in the composition of the phospholipid fatty acids in the intestinal muscle during the absorption of fat. The data in Table II seem to indicate such a change; not only is the average I.N. lower for the cats absorbing the oil, but a close scrutiny of the individual experiments shows that there is a fair agreement between the I.N.'s of the phospholipid fatty acids in the mucosa and muscle of the same intestine, irrespective of the conditions. These facts were interpreted to mean that absorbed fatty acids are transformed into phospholipid in both the mucosa and the muscle of the small intestine.

Since it is inconceivable that the intestinal muscle is concerned in the process of fat absorption, it was concluded that the observed change in the composition of the phospholipid fatty acids was due to a continuous metabolism of phospholipid in both the mucosa and the muscle. This conclusion is not in accord with the prevailing opinion that the phospholipids are not active metabolites involved in the production of energy, but are vital constituents of living protoplasm (Mayer and Schaeffer, 1913).

Series III. Effect of Olive Oil on the Phospholipid Fatty Acids in Rat Skeletal Muscle.

In view of the resemblance of the results on the intestinal muscle to the decisive results on the mucosa, it seemed worth while to extend the investigation to a study of the effect of fat absorption on the phospholipid fatty acids of the skeletal muscle. As cats were scarce at this time, white rats were used. The rats were killed by a blow on the head, and the skin, head, tail, feet, and viscera were removed; the carcass was passed twice through a meat grinder. A convenient amount of the mixture of hashed

muscle and bone was weighed out and extracted in the usual manner. The change in the method of precipitating the phospholipid which was mentioned in the "Experimental" section was adopted at this stage in the investigation. Henceforth, either magnesium chloride or cadmium chloride was used to obtain more complete precipitation of the phospholipids. Also, the term "phospholipid" was extended to include all of the acetone-insoluble portion.

The results of the experiments on rat skeletal muscle are set forth in Table III. Olive oil was used because its I.N. of 86 is low in comparison to the I.N. of the phospholipid fatty acids. The group of experiments marked Series S was conducted first. The data obtained show very clearly that absorbed olive oil has no effect on either the amount or composition of the phospholipid fatty acids in skeletal muscle, in contrast with the results on intestinal muscle. Since the rats were quite inactive most of the time while the intestinal muscle is active during absorption, it was thought that the difference in activity was a possible explanation for the disagreement in the results. Therefore, another set of experiments was conducted in which both the control rats and those absorbing olive oil were exercised continuously in a rotating cage for 8 hours before death. However, the results of these experiments (Series E, Table III) agree in every respect with those of Series S. From these results it may be concluded that neither fat absorption nor moderate exercise has any effect on the amount or composition of the phospholipid fatty acids in the skeletal muscle of the rat.

Series IV. Effect of Cod Liver Oil on the Phospholipid Fatty Acids of the Mucosa and Muscle of the Same Intestine.

Naturally these decisive results on rat skeletal muscle served to cast suspicion on the reality of the apparent change in the phospholipid fatty acids of intestinal muscle. Since cod liver oil had given such consistent results in previous experiments, it was decided to use this oil in a new series of experiments on the mucosa and muscle of the small intestine. The results are set forth in Table IV. As no attempt was made to maintain the cats on a constant diet (the only precaution having been to use two cats from the same cage for each pair of experiments), there is a greater variation than usual in the I.N.'s of the phospholipid fatty acids

in the mucosæ of the controls. However, the data on the mucosæ show an exceedingly good agreement with the former results obtained with cod liver oil (Table I). These results show conclusively that there is a change in the composition of the phospholipid fatty acids in the absorbing mucosa without any appreciable change in amount.

TABLE IV.
Fatty Acids of Acetone-Insoluble Lipids from Small Intestine.

Experimental conditions.	Cat No.	Mucosa.			Muscle.		
		Weight in:		Iodine No.	Weight in:		Iodine No.
		Moist mu- cosa.	Dry mu- cosa		Moist mus- cle	Dry mus- cle	
		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
Control cats, post-absorptive.	27*	1 23	7 08	97	0 57	2 68	99
	29	0 97	6 32	97	0 55	2 70	96
	33	1 03	8 00	88	0 52	2 28	93
	35	1 13	7 76	89	0 69	4 00	96
	37	1 27	8 05	97	0 62	3 63	101
Average		1.13	7.44	94±2.1	0.59	3.06	97±1.4
Fed cod liver oil:							
6 hrs. before death.	28*	1 28	7 72	106	0 63	3 50	98
7 " " "	30	1 19	7 27	110	0 63	3 16	98
6 " " "	34	1 08	7 24	102	0 57	2 85	100
5 " " "	36	1 15	7 77	107	0 69	3 60	101
5 " " "	38	1 06	6 03	110	0 69	3 75	100
Average.....		1.15	7.21	107±1.5	0.64	3.37	99±0.6

* Cats 27 and 28, 29 and 30, etc., were kept in the same cage, fed on the same food, and killed on the same day. The food consisted mainly of meat scraps.

The results on the intestinal muscle are not consistent and decisive as are those on the mucosa. Two experiments out of five show that the phospholipid fatty acids have a higher I.N. in the muscle of the absorbing intestine than in the corresponding control; in the other three experiments there is no difference. The net result is that the average I.N. in the cats absorbing cod liver oil is slightly higher than in the controls but not sufficiently so to

be regarded as significant. On the other hand, these experiments prove beyond a doubt that the composition of the phospholipid fatty acids in the intestinal muscle is influenced by the type of diet fed over a considerable period of time. Thus, in the cats which were fed on meat scraps the average I.N. is 97 ± 1.4 , while in the cats which were fed on beef kidney the average I.N. is 108 ± 2.9 . In view of this effect of diet it seems unwise to conclude that the composition of the phospholipid fatty acids in intestinal muscle is not at all influenced by absorbed fat. Nevertheless, it is apparent that the former conclusion that fat absorption has the same effect on the phospholipid of the mucosa and muscle of the small intestine is quite erroneous. There is now no doubt that *the incorporation of absorbed fatty acids into the phospholipid of the mucosa is a specific process*. But, as yet, the evidence does not permit the conclusion that this specific process in the mucosa is an integral step in the resynthesis of neutral fat.

Series V. Time Factor in Transformation of Absorbed Fatty Acids into Phospholipid. Effect of Cod Liver Oil and Olive Oil on the Phospholipid Fatty Acids of Intestinal Mucosa and Muscle, Skeletal Muscle, and Liver of the Same Cat.

Up to this time all of the absorption experiments were terminated at the 5th to the 7th hour (usually the 6th) after the administration of the fat. It seemed desirable to know: (1) how soon the transformation of fatty acids into phospholipid takes place; (2) how long the altered composition of the phospholipid fatty acids of the mucosa is maintained after fat absorption has been completed; and (3) what influence such a fat as olive oil (I.N. 86) has on the I.N. of the phospholipid fatty acids. Furthermore, it is evident that the change in composition of the phospholipids of the mucosa during fat absorption is similar in many respects to the change which has been observed to take place in the phospholipids of the liver. Joannovics and Pick (1910) observed that during the absorption of cod liver oil by dogs there is a pronounced increase in the I.N. of the phospholipid fatty acids in the livers, without any appreciable change in amount. It was decided to compare in the same cat, the effect of fat absorption on the composition of the phospholipid fatty acids in the intestinal mucosa, intestinal (smooth) muscle, skeletal (striated) muscle,

and the liver. A number of cats were prepared for these experiments by feeding for several weeks on a constant diet of hashed lean beef, bread, and onions. The results are set forth in Table V.

TABLE V.

Comparative Effect of Absorbed Fat on Fatty Acids of Acetone-Insoluble Lipids of Various Tissues.

Cat No.	Time of feeding before death.	Mucosa.			Intestinal muscle.			Skeletal muscle.			Liver.		
		Weight in:		Iodine No.	Weight in:		Iodine No.	Weight in:		Iodine No.	Weight in:		Iodine No.
		Moist tissue.	Dry tissue.		Moist tissue.	Dry tissue.		Moist tissue.	Dry tissue.		Moist tissue.	Dry tissue.	
Control cats.*													
	hrs.	per cent	per cent		per cent	per cent		per cent	per cent		per cent	per cent	
1	24	1.16	7.57	89	0.62	3.07	90	0.68	3.61	107			
4	24	1.27	8.46	85	0.81	4.68	89	0.56	2.89	104	1.65	6.87	105
6	24	1.41	8.75	88	0.75	4.16	95	0.61	3.14	113	2.08	7.98	120
9	48	1.36	7.20	89	0.62	3.41	94				2.50	11.50	120
Average.....		1.30	8.00	88	0.70	3.83	92	0.62	3.21	108	2.08	8.78	115
Cats fasted 24 hrs. and then fed 13 to 14 gm. of cod liver oil.*													
2	2	1.16	8.37	94	0.61	3.06	84	0.59	3.06	107	1.99	8.74	115
3	4	1.03	7.07	98	0.50	2.72	96	0.65	3.40	109	2.38	11.44	131
5	6	1.15	8.17	100	0.59	3.22	94	0.63	3.41	106	2.23	10.23	134
7	8	1.19	8.42	102	0.59	3.45	94	0.58	2.99	110	1.98	8.50	140
8	24	1.29	8.79	99	0.78	4.43	94				2.04	10.72	159
10	46	1.52	8.94	101	0.54	2.72	95				2.48	12.08	159
13	75	1.54	7.82	94	0.62	3.23	94				2.58	11.32	129
11†	9	1.29	9.24	81	0.61	3.34	89				1.86	7.68	110
12‡		1.16	8.23	94	0.58	3.19	94				2.48	11.00	142
Average.....		1.26	8.34		0.60	3.26	93	0.61	3.22	108	2.22	10.19	

* All cats in this series were fed on a standard diet of 3 parts of boiled lean beef (the fat which rose to the surface was removed) and 1 part of bread for at least 2 weeks before use.

† Fed olive oil.

‡ Fed cod liver oil at 48 hours, and olive oil at 7 hours before death.

The data on the intestinal muscle agree with those in Table IV. Likewise the data in Table V agree with the previous observations

on the rat (Table III) in showing decisively that absorbed fat has no effect on either the composition or the amount of phospholipid fatty acids in skeletal muscle. It is interesting to note that the average of 0.61 per cent for the amount of the phospholipid fatty acids in the moist smooth and skeletal muscle of normal cats is the same as that found by Terroine and Belin (1927) for the amount of fatty acids in the so called *élément constant* of the muscles of rabbits which had died of inanition. On the other hand, it must be pointed out that the well marked influence of diet on the I.N. of the phospholipid fatty acids in cat muscle does not agree with the observations of Terroine and Belin that the type of fat fed previous to the fasting period had no effect on the I.N. of the fatty acids in the *élément constant*. With respect to the liver the data agree, in the main, with the results of Joannovics and Pick (1910).

The facts concerning the influence of fat absorption on the phospholipid fatty acids of the intestinal mucosa, as revealed in Tables I, II, IV, and V, may be summarized as follows: (1) The *amount* does not change. Thus, if the data set forth in Tables IV and V are combined, the average amount of phospholipid fatty acids in the mucosæ of the control cats is 1.20 ± 0.05 per cent of the moist weight and 7.69 ± 0.19 per cent of the dry weight, while for the cats which were absorbing fat the values are 1.22 ± 0.04 per cent and 7.92 ± 0.23 per cent, respectively. (2) The fatty acids of the ingested fat are incorporated into the phospholipid of the mucosa. (3) The fatty acids of cod liver oil are still present in the phospholipids of the intestinal musosa of a cat which has been fasted for 3 days after administration of 13 gm. of the oil. (4) If olive oil is administered to a cat which was fed cod liver oil 41 hours previously, the I.N. of the phospholipid fatty acids is lower than in a cat which was fasted for 2 days after being fed cod liver oil, and higher than in a cat which was fed olive oil 24 hours subsequent to the standard rations (experiments on Cats 11, 12, and 13, Table V).

Interpretation of Results.

Although the absence of any increase in the amount of phospholipid fatty acids is good evidence that the observed change in I.N. is not due to contamination by the ingested fat, final proof

was obtained by adding 1 cc. of cod liver oil to the alcoholic extract of the mucosa of Cat 35 (Table IV). The effect is negative. Furthermore, the absence of a change in the composition of the phospholipid fatty acids in muscle is decisive proof that the inclusion of blood phospholipid is *not* responsible for the phenomenon observed in the intestinal mucosa.

The incorporation of absorbed fatty acids into the phospholipid of the intestinal mucosa may indicate: (1) that this process is an essential step in the resynthesis of the absorbed fatty acids into neutral fat; (2) that there is a neogenesis of phospholipid in the mucosa; (3) that the phospholipid of the mucosa is being continuously burned and immediately replaced by synthesis from the available fatty acids.

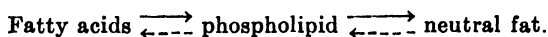
The idea that phospholipid is an intermediary metabolite in the combustion of fat is not supported by the known facts. If it be so, phospholipid metabolism must be characteristic of only certain tissues, such as the intestinal mucosa and the liver, since both Leathes (1909) and Shioji (1924) have shown that even the total fatty acids of the heart and kidney are only slightly influenced by food fat, and the present work shows that the phospholipid fatty acids of both smooth and skeletal muscle are not affected by absorbed fat. Furthermore, it is difficult to reconcile the conception of phospholipid metabolism within the mucosa with the absence of any decrease in amount during fasting, and especially with the presence of cod liver oil fatty acids in the phospholipid after 3 days of fasting.

The possibility that neogenesis of phospholipid from absorbed fatty acids within the intestinal mucosa is responsible for the increase in blood phospholipid during fat absorption has already been considered by Eckstein (1924-25). He believed that, if this were true, there should be an increase in the phospholipid of the thoracic lymph during fat absorption, just as there is an increase in neutral fat. His experiments showed that there is no change in the phospholipid content of the lymph during the absorption of olive oil. Furthermore, Zucker (1919) has shown that there is no difference in the phospholipid content of the portal and jugular blood, either before or during the absorption of fat. These observations, together with such facts as the marked stability of the amount of the phospholipid fatty acids, both during fasting and

during fat absorption, and the well marked uniformity of the change in I.N., all militate against the idea that new phospholipid is synthesized in the intestinal mucosa.

The hypothesis that absorbed fatty acids are transformed into phospholipid as an essential step in the resynthesis into neutral fat may be stated provisionally as follows:

Within the epithelial cells of the intestinal mucosa there is a "specific" phospholipid which occupies an intermediary position between fatty acids and neutral fat, as represented by the equation



It is an invariable characteristic of this process that the *amount* of phospholipid remains constant. As soon as fatty acids are absorbed into the epithelial cells, molecules of the "specific" phospholipid react with the free fatty acids (or soaps) to form neutral fat; immediately, however, the phosphoric acid-base complex unites with absorbed fatty acids and glycerol to form phospholipid, thereby maintaining the amount constant. The synthesized molecules of neutral fat, being insoluble in the cell fluids, coalesce and form the droplets which are so typical of the process of fat absorption. In this manner the neutral fat is made inactive, and the reaction proceeds until all of the absorbed fatty acids have been synthesized into neutral fat.

The idea that phospholipid may be transformed into neutral fat is not new. Meigs, Blatherwick, and Cary (1919) have presented evidence to the effect that phospholipid is removed from the circulating blood and transformed into milk fat by the secreting mammary glands. Recently, Theis (1928) has observed that the injection of insulin into rabbits causes a decrease in the phospholipid and an increase in the neutral fat in the liver. It is interesting to note that Frank in 1898 made the suggestion that the resynthesis of fat was accomplished by the interaction of lecithin and soaps.

The following deductions from the hypothesis offer satisfactory explanations for many of the observed facts:

1. The "specific" phospholipid within the epithelial cells must comprise a more or less fixed proportion of the total phospholipid of the intestinal mucosa.
2. At such a time as fat absorption is going on in every

epithelial cell of the whole mucosa, the constituent fatty acids of the "specific" phospholipid must consist entirely of the absorbed fatty acids. These two deductions explain why it is that the change in I.N. following the ingestion of cod liver oil reaches an apparent maximum within 5 to 7 hours, and why there is such a well marked uniformity in the results of the various experiments. In experiments on Cats 37 and 38 (Table IV) the mucosæ were divided into two approximately equal lengths and the upper and lower parts of each mucosa were studied separately. Although there were appreciable differences in the percentage amounts of the phospholipid fatty acids in the two parts of the same intestine, the I.N.'s were identical. This indicates that in the experiment on Cat 38 there was a complete involvement of the "specific" phospholipid in the process of fat resynthesis at the 5th hour following the ingestion of cod liver oil.

3. After fat absorption has been completed, the constituent fatty acids of the "specific" phospholipid must consist of the most recently absorbed fatty acids. Fasting should have no effect on the composition of the fatty acids in the "specific" phospholipid, except in so far as the lipids of the bile, pancreatic juice, and succus entericus are absorbed. The observations made in the last series of experiments (Table V) agree with this deduction. Thus it was found that even after fasting for 1, 2, and 3 days following the administration of the oil, the phospholipid fatty acids of the mucosa contained a percentage of cod liver oil fatty acids almost as high as during absorption.

It is hoped that further work will clarify some of the observed facts which cannot as yet be explained by the hypothesis. For instance, it is apparent that the type of preliminary diet has an influence on the composition of the phospholipid fatty acids in the mucosa. That this influence is not merely due to the absorption of the characteristic fatty acids in that diet is shown by the fact that cod liver oil does not produce the same I.N. in the different series of experiments. Furthermore, it is not known what proportion of the total phospholipid of the mucosa consists of the "specific" phospholipid of the epithelial cells. Since olive oil (I.N. 86) produced a lower I.N. in the phospholipid fatty acids than the preabsorptive I.N. of 88, it must be concluded that the fatty acids of the "specific" phospholipid had a higher I.N. than

the average for the whole mucosa. If one uses the I.N.'s of the phospholipid fatty acids in the controls and after the absorption of olive oil and cod liver oil (Table V), it can be calculated that the fatty acids of the "specific" phospholipid comprise 25 per cent of the total and, in the controls, have an I.N. of 118, the fatty acids of the phospholipids in the remainder of the mucosa having an I.N. of 78. However, this calculation involves the assumption that the latter remains constant during fat absorption, an assumption which is not yet justifiable. The fact that the absorption of olive oil 41 hours after cod liver oil does not give as low an I.N. for the phospholipid fatty acids as when olive oil is absorbed 24 hours after the standard ration cannot be explained by the above hypothesis.

Nevertheless, it is believed that most of the evidence supports the hypothesis that the observed transformation of absorbed fatty acids into phospholipid is an essential step in the resynthesis of neutral fat within the epithelial cells of the intestinal mucosa.

SUMMARY.

During the absorption of fat: (1) There is a pronounced change in the *composition* but no change in the *amount* of the phospholipid fatty acids of the intestinal mucosa and of the liver. (2) There is no significant change in either the amount or the composition of the phospholipid fatty acids of intestinal (smooth) muscle or skeletal (striated) muscle.

It is suggested that absorbed fatty acids are transformed into phospholipid within the intestinal mucosa as an essential step in the resynthesis of neutral fat.

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THE DETERMINATION OF TRUE SUGAR IN BLOOD.

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It is generally appreciated that the "sugar" of blood, as determined in "protein-free" blood filtrates by any of the current reduction methods, includes reducing substances other than the true sugar. Furthermore the identity of all of the true sugar is perhaps not yet fully established as glucose. It is therefore to be expected that different copper or other reagents, all standardized as to their *glucose* equivalents, will yield each a different result when applied to such a mixture of glucose (?) and other unidentified reducing substances as is contained in blood filtrate. That expectation is fully realized in the work of a number of investigators; it is indeed surprising that results on the same blood by different methods do not differ more than they do.

Assuming the true sugar of blood to be glucose, two essentially different plans may be followed in designing an analytical process for its determination. The simplest and most direct plan would obviously be to choose or construct some highly selective reagent which responds only to glucose, and fails to react with such other reducing substances as may be present in blood filtrates prepared by tungstic acid precipitation or similar precipitations now in use. The second plan would be the removal of the interfering reducing substances, leaving only the true sugar in the filtrates.

With a few exceptions the attempts to determine true sugar have so far followed the first plan. Two types of selective reagents have been used: the selective destruction of the true sugar by yeast fermentation or by glycolysis and its determination by the loss of reducing power toward sensitive reagents which react also with reducing "non-sugars;" and the construction of less sensitive copper reagents designed to be reactive only toward the true sugar. Results by the use of yeast should be decisive, but were not wholly satisfactory until Somogyi emphasized the importance of pre-

liminary purification of the yeast suspensions and described the optimum conditions for their use. The earlier conflicting results with yeast are reviewed by Van Slyke and associates (1) and by Somogyi (2). By the Somogyi technique it seems probable that the true sugar may be determined with accuracy. The second type of selective reagent is represented by those of Benedict (3) and Folin (4), all of which however show the presence of reducing substances in blood filtrates after fermentation, and therefore give with unfermented filtrates results which though lower are perhaps only relatively *less erroneous* than are obtained with more sensitive reagents. To be sure Benedict reports data ((3) Table I, p. 468) which show satisfactory determination (± 3 mg. per cent) by his latest (1928) reagent of glucose (100 mg. per cent) added to previously fermented blood filtrates; though before the addition of the sugar the same fermented filtrates with the same reagent gave a reduction corresponding to from 6 to 14 mg. per cent. From this fact one may conclude either that the *sum* of non-fermented substances *plus* added sugar is not completely determined, or that *in the presence of sugar* (though not in its absence) the non-fermented substances are inactive toward the reagent, and that the results represent only the added glucose or the true sugar. The latter is Benedict's interpretation, and may be the correct one in spite of the somewhat remarkable assumption that *the presence of sugar somehow prevents the oxidation of the non-sugars*. In this connection it may be noted that the *difference* between the reducing values before and after fermentation, presumably the true sugar, in the data reported by Benedict, are with Benedict's reagent from 6 to 24 mg. per cent (average 15.6) *lower* than by the Folin-Wu reagent. Since both reagents are standardized against glucose, the decrease of reduction by fermentation should be identical unless (1) the true sugar is *not* glucose but some other sugar (or mixture) having different reducing power toward the two reagents or (2) unless the presence of other substances in the filtrates decreases the sensitiveness of the Benedict reagent toward the sum of true sugar and reducing non-sugars by an amount about equal to the reducing power of the non-sugars. It can hardly be supposed that the Benedict reagent is insensitive toward the non-sugars in view of the fact that when the true sugar is removed by fermentation the reagent is reduced by them, though only about half as much (in terms of glucose) as with the Folin-Wu reagent. It seems impossible at present to decide between these two possibilities.

For the second plan mentioned above, the removal of interfering substances preliminary to sugar determination, precipitation by mercury salts has proved effective. Introduced in 1887 by Johnson (5), who used mercuric chloride with urine, the process was improved by Patein and Dufau (6) who employed acid mercuric nitrate neutralized by alkali. The Patein-Dufau solution has been used with urine and blood by a number of later workers, Deniges (7), Benedict and Osterberg (8), Shaffer and Hartmann (9), Ronzoni and Wallen-Lawrence (10), Harned (11), and Bierry and Voskresensky (12), several of whom have shown that under certain conditions glucose is not lost and that the precipitation does not interfere with subsequent determination of sugar. Harned has compared results for blood

sugar by the Folin-Wu colorimetric method in filtrates from mercuric nitrate-bicarbonate precipitation with results in tungstic acid filtrates by the Folin-Wu and Benedict (1925) (13) methods. His results by the Folin-Wu reagent¹ on mercury filtrates of twenty-six normal and diabetic human bloods are 12 to 28 mg. per cent lower than on the tungstic acid filtrates.

If all of the reducing substances other than true sugar are removed by mercuric nitrate precipitation, the results by any *single* copper reagent on such filtrates would agree with the *loss* of reducing power by fermenting tungstic acid filtrates. And similarly the *decrease* of reduction caused by mercury precipitation should agree with the *residual* reduction after fermentation. These hypothetical relations may be clearer from the following.

Tungstic acid filtrate (A)	—	mercury filtrate (B)	=	reducing substance removed by Hg. (C)
Tungstic acid filtrate (A)	—	tungstic acid fil- trate after fermen- tation (D)	=	fermentable reduc- ing substances, true sugar. (E)

If the total observed reduction is expressed in terms of mg. per cent of glucose in the original blood and the loss on fermentation (E) taken as the most probable value for the true sugar, the question is, with any single copper reagent does $B = E$ and $C = D$? According to the work of Somogyi (2) the value of D, the "reducing non-sugars" left after fermentation (with his modification of the Shaffer-Hartmann reagent), in 52 human bloods (whole bloods) averages 26 mg. per cent, the extremes being 31 and 23. By a slightly different reagent Dr. Somogyi informs us he finds an equally constant value at 20. From Benedict's data ((3), p. 468) we find for the same fraction in ten bloods, by the Folin-Wu reagent 20 ± 3 and by the Benedict reagent 11.7 ± 3 . Folin and Svedberg (14) report values in thirty-five diabetic bloods, with the Folin-Wu reagent averaging 20 ± 5 , and with the Folin reagent 9 ± 4 . As would be expected the glucose equivalent of the non-

¹ Harned's results by the Benedict reagent on fifteen tungstic acid filtrates averaged only 5 mg. per cent higher than the Folin-Wu results on mercury filtrates, indicating nearly correct results by the Benedict reagent on filtrates containing the non-sugar.

sugars varies with reagents used and comparison must be limited to the same reagent. Taking 20 mg. per cent as the value of *D* (the non-fermentable reducing substance in tungstic acid filtrates) by the Folin-Wu reagent, we may compare Harned's data on the *decrease* of reduction toward the same reagent caused by mercuric nitrate precipitation. His values range from 13 to 26 with an average of about 20 mg. per cent. The agreement indicates that the amount *left* by yeast approximately equals the amount precipitated by mercury salts though the variation of 13 to 26 is somewhat greater than found by Somogyi with his own reagent. The same comparison may be made of the *Somogyi reagent* value of 26 ± 4 with the results by that reagent following mercury precipitation recorded by Ronzoni and Lawrence. In a series of eight bloods the *difference* between results on tungstic acid and mercuric nitrate filtrates varies but little from 13 mg. per cent. The agreement in this case between 26 mg. of non-fermented and 13 mg. removed by mercury is less satisfactory and suggests that perhaps the precipitation of non-sugars by mercury was incomplete.

According to our experience in carrying out this comparison, discrepancies of this sort are not uncommon, and are perhaps due to unavoidable variations in the alkalinity of the solutions when bicarbonate or hydroxide is used for neutralization in the mercuric nitrate precipitation. The completeness of precipitation of the non-sugars varies with the reaction at which the precipitation takes place. We therefore sought means of obviating this difficulty in order that a practical procedure might be had, giving consistent and dependable results. With the slight modifications now introduced the mercuric nitrate precipitation yields filtrates, the reducing power of which, in practically all human bloods so far analyzed, agrees closely with the true sugar as indicated by loss of reducing power on yeast fermentation. As a rule *B* equals *E* in the above equations. The preparation of the mercury filtrates is little more laborious than the preparation of tungstic acid filtrates and requires less time and manipulation than Somogyi's yeast method. The procedure is therefore recommended as a method for the direct determination of true blood sugar; direct, in the sense of preliminary removal of all interfering substances.

In some bloods, notably of pig and sheep, the mercury filtrates

contain a few mg. per cent more reducing power than corresponds to the fermentable sugar. The identity of these non-fermentable substances is not indicated by our data, but may be non-fermentable sugars such as appear to be present in many normal urines as suggested by Greenwald, Gross, and Samet (15) and Höst (16), since it is to be expected that the same reducing substances present in urine are to be found also in blood, though not in the same relative amounts. The bloods of two human subjects, a case of hypertension with uremia and another of "malignant hypertension" with uremia have also given mercury filtrates in which the reducing power was several mg. per cent higher than corresponds to the true sugar by fermentation. This clue is being followed but so far we have found no other cases.

Procedure.

We shall describe two different methods of carrying out the mercury precipitation, in both of which neutralization is accomplished by solid barium carbonate. The result of this method of neutralization is that a satisfactory reaction is automatically attained and variations of alkalinity which occur when NaOH or NaHCO_3 are used, are thereby avoided.

The first process utilizes the Patein-Dufau mercuric nitrate reagent, which in the second is substituted by a solution of mercuric sulfate in sulfuric acid. The latter possesses the distinct advantage in that the precipitation of non-sugars is if anything more complete, and that all of the constituents of the reagent are removed, leaving only the electrolytes of the blood in the filtrate. The sulfate as well as the mercury is removed by treatment with the barium carbonate. A decided advantage of the mercuric sulfate method from a practical standpoint is the rapidity with which it can be carried out, especially if the zinc method of removing mercury and rapid filter papers are used. In all respects we consider the mercuric sulfate method superior to the nitrate with the minor exception that it requires more barium carbonate for neutralization.

For determination of the reducing power of all blood filtrates a Shaffer-Hartmann copper reagent, as modified by Somogyi, has been used. The reagent is a second unpublished modification by

Dr. Somogyi, of the following composition, with which a table of glucose values was constructed by him with pure glucose.

	gm. per l.
Sodium carbonate, anhydrous.....	20
“ bicarbonate.....	25
Rochelle salt.....	25
Copper sulfate, crystalline.....	7
Potassium oxalate.....	5
“ iodide.....	10
“ iodate.....	0.8

Mercuric Nitrate Method.

Mercuric Nitrate Reagent of Patein and Dufau.—220 gm. of mercuric oxide are dissolved in small portions in 160 cc. of concentrated nitric acid. The solution is boiled, cooled, 60 cc. of 5 per cent sodium hydroxide added, diluted to 1000 cc., and filtered through asbestos. It is preserved in a brown bottle.

5 cc. (1 volume) of blood are laked in 40 cc. (8 volumes) of water in a 125 to 150 cc. Erlenmeyer flask fitted with a rubber stopper. 5 cc. (1 volume) of the mercuric nitrate reagent are added slowly from a burette with shaking. The flask is stoppered and vigorously shaken to break up the gel. Approximately 3 gm. of precipitated barium carbonate² are added and the flask rapidly rotated for a few seconds until most of the CO₂ has escaped. The flask is then stoppered, shaken hard, and opened to release carbon dioxide. Shaking is repeated until there is no further pressure developed in the flask. Neutralization requires 2 or 3 minutes and is indicated by the absence of pressure in the flask after stoppering and shaking, the visible presence of an excess of barium carbonate, and the fact that red litmus paper moistened by the liquid is slowly turned slightly blue. The precipitated material is poured upon a rapid filter³ and the filtrate (16 to 18 cc.) collected in a 125 to 150 cc. Erlenmeyer flask. 1 drop of H₂SO₄ (1:1)

² c.p. and pure “precipitated” BaCO₃. We have used the preparations from Mallinckrodt. The physical condition of the substance varies considerably, which affects its neutralizing power, especially in the case of mercuric sulfate precipitation. A very finely divided material is preferable.

³ For the most exact work the filter papers should be washed with hot water and dried or tested for their content of extractable reducing substances as suggested by Benedict.

and 0.3 gm. of anhydrous Na_2SO_4 * are then added to the filtrate and the mercury precipitated by treating with H_2S (moistened by bubbling through water) for a half minute. Excess hydrogen sulfide is blown off by a rapid current of air, likewise moistened by bubbling through water. This requires about 30 seconds. A drop of 10 per cent CuSO_4 is added to insure the removal of the last traces of H_2S and the solution filtered or centrifuged; if centrifuged the clear supernatant liquid is passed through a small filter to remove suspended particles. The preparation of the filtrate takes about 45 minutes. 5 cc. portions are accurately measured into 25×200 mm. test-tubes. 2 drops of phenol red (0.05 per cent in water) are added to each tube, followed by 0.5 N NaOH drop by drop from a medicine dropper until the red color of the indicator appears. Ordinarily 3 to 6 drops of the alkali are required to neutralize a 5 cc. portion of filtrate. 5 cc. of the Shaffer-Hartmann reagent are added and the sugar determination executed in the usual way.

Some points call for brief comment. An excess of barium carbonate above 3 gm. is of no consequence except that the volume of filtrate obtained is reduced. A larger quantity of filtrate, 5 to 8 cc., is obtained and the time required is possibly less when the precipitate is removed by centrifugation. 0.4 to 0.5 gm. of Na_2SO_4 should be added to the filtrate in this case. 1 drop of the H_2SO_4 is sufficient.

The quantity of filtrate obtained and the rate of filtration are increased by vigorously shaking the precipitated mixture with short quick jerks.

A large excess of sodium sulfate should be avoided because as shown by Somogyi (17) high salt concentration causes an increase in reduction values. The salt concentration of filtrates prepared as above outlined is close to 2 per cent and does not perceptibly affect the determinations with sugar concentrations under 100 mg. per cent. With concentrations of 200 mg. per cent the salt error is about $+3$ mg. per cent.

The pH of filtrates immediately after precipitation and filtration

* The Na_2SO_4 must not be added to the blood-barium carbonate mixture before filtration. When this is done barium carbonate reacts with Na_2SO_4 forming BaCO_3 which raises the pH of the solution into the distinctly alkaline region and erratic sugar values are obtained.

was determined colorimetrically. In a series of seven filtrates prepared consecutively from beef blood six had a pH of 6.3 and one of 6.2. If the filtrates be prepared and allowed to stand exposed to the air for a time, irregular and higher pH values are found which are the result of the loss of carbon dioxide. After standing the pH may be above 7. It is likely that at the time of neutralization by barium carbonate the pH is rather less than 6.3 because a small amount of CO_2 escaped before the tests were made. The significant fact is that a uniform pH is automatically obtained in the precipitation, thereby insuring uniform removal of interfering substances.

Sugar determinations on fermented mercury filtrates of beef blood, with and without added glucose, were made. The effect of adding the approximate quantity of salts found in mercuric nitrate-barium carbonate filtrates to pure glucose solutions was also determined. The results show that the fermented mercury filtrates contained no reducing non-sugar or non-fermentable sugar detectable by the reagent used and that added glucose is quantitatively recovered when correction for the salt error is made. For example 198 mg. per cent of glucose were added to the fermented mercury filtrate and 201 mg. per cent found. 197 mg. per cent of glucose were added to a salt solution (1.5 per cent NaNO_3 + 0.5 per cent Na_2SO_4) and 200 mg. per cent found. The salt error, 3 mg. per cent, subtracted from 201 gives a recovery of 198 mg. per cent, the sugar added to the fermented filtrate. The salt error for a glucose solution of 63 mg. per cent was found to be about + 1 mg. per cent. These salt errors, certainly in the lower blood concentrations, are within the limits of experimental error for the method as applied to blood and no corrections have been made for them in the results of Tables I to III. They are probably partly balanced by similar errors in the Folin-Wu filtrates.

Mercuric Sulfate Method.

*Mercuric Sulfate Reagent.*⁴—The reagent consisted of a 30 per cent solution of mercuric sulfate in 10 per cent sulfuric acid. A

⁴ The reagent is best prepared by adding the sulfate, in small portions with shaking, to a volume of 10 per cent H_2SO_4 equal to about 0.9 of the final volume. After all of the sulfate is dissolved the solution is diluted to the required volume with more of the acid.

slight precipitate of the basic sulfate generally forms from which the solution is decanted.

Procedure.

5 cc. of blood (1 volume) are laked in 50 cc. of water (10 volumes) and 5 cc. (1 volume) of acid mercuric sulfate added, with shaking, from a burette. The flask is stoppered and thoroughly shaken to break up the gel. 9 to 10 gm. of precipitated barium carbonate are added, the flask rotated for a few seconds until most of the carbon dioxide has escaped, and then stoppered and shaken. Carbon dioxide is released and the flask again stoppered and shaken. This is repeated until no further pressure develops in the flask and the mixture does not turn blue litmus red (neutral to litmus). If neutralization does not take place promptly, add 1 to 2 gm. of additional barium carbonate. The mixture is poured upon a rapid filter and traces of barium and mercury removed from the filtrate in either of two ways. One procedure is to add a drop of saturated sodium sulfate to the filtrate (20 to 22 cc.), and a pinch of zinc dust, followed by shaking for a few seconds. The zinc and trace of barium sulfate are filtered off on a rapid filter and the filtrate is ready for analysis. This method is recommended for use with the modified Shaffer-Hartmann reagent which we have employed. It has been found that the hydrogen peroxide formed in shaking the filtrates with Zn dust causes erroneous results upon heating with Shaffer-Hartmann reagents containing no iodide or insufficient quantities of it. The reagent of the composition given above is entirely unaffected by the peroxide and gives the same results with the zinc and hydrogen sulfide filtrates. It also gives the true sugar values on pure glucose solutions treated with either mercury and zinc or with hydrogen peroxide. Upon standing these zinc-treated filtrates develop a slight precipitation, presumably of zinc carbonate. It causes no trouble. The second method recommended for the removal of mercury is the usual treatment with hydrogen sulfide. 1 drop of concentrated sulfuric acid is added to the filtrate, moistened hydrogen sulfide passed through it for a half minute, and the latter removed by a current of moist air. 1 drop of 10 per cent CuSO_4 is added and the precipitate filtered off on a rapid filter. The filtrate is neutralized before sugar determination in the manner

indicated for mercuric nitrate filtrates. It is to be noted that the dilution is 1:12 in the mercury sulfate filtrates instead of the customary 1:10.

The mercury sulfate filtrates of normal blood are nitrogen-free. A study is being made of the nitrogen in these filtrates of pathological bloods.

Experiments demonstrated that the fermented mercury sulfate filtrates of normal bloods contain no reducing substances detectable by the sugar reagent used and that added sugar is quantitatively recovered from such filtrates. There is no salt error. For example, 199 mg. per cent of glucose were added to fermented mercury sulfate filtrate of beef blood and 200 mg. per cent found.

Table III gives sugar values obtained on mercury sulfate filtrates of various bloods in comparison with those by fermentation and in a few cases by mercuric nitrate.

DISCUSSION.

The data presented in Table I give a comparison of the sugar content of human blood as obtained directly on the mercury filtrates and by Somogyi's fermentation procedure. It is assumed that the loss on fermentation represents the true sugar. With the exception of the bloods from two pathological subjects, the agreement between the true sugar values and those by direct determination is within the accuracy obtainable with the reagent. In most cases the results differ in either direction only 2 or 3 mg. per cent, in four cases out of twenty-six the mercury filtrate result is 5 mg. per cent higher. With the eleven normal bloods the average by the two methods is identical, and including the diabetic and nephritic bloods (excluding the two cases, Samples 21 and 28) the averages are 103 and 103.6 mg. per cent.

In the samples of Series 21 from a case of cardiac hypertension with high blood non-protein nitrogen we found an interesting exception. There was some reducing (or iodine-absorbing) substance present in the blood of this patient which was not precipitable by the mercury treatment and which was absent from the other bloods examined, with the possible exception of Sample 28. The fact that the patient of Series 21 was completely digitalized at the time all bloods were drawn suggested that possibly the digitalis was contributing directly or indirectly to the reducing

TABLE I.
Human Blood.

Sample No. (1)	Description of blood. (2)	Sugar, F.W.* (3)	Reducing non-sugar, F.W.Y.* (4)	True sugar, (3)-(4). (5)	Sugar, Hg(NO ₂)+ BaCO ₃ . (6)
		mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	Normal human.	119	25	94	96
2	" "	93	19	74	75
3	" "	88	25	63	62
4	" "	98	21	77	75
5	" "	87	18	69	70
6	" "	87	19	68	66
7	" "	91	17	74	76
8	" "	102	18	84	85
9	" "	92	19	73	71
10	" "	92	16	76	77
11	" "	93	24	69	68
12	Mixed hospital.	158	19	139	142
13	" "	129	17	112	112
14	" "	114	20	94	99
15	" "	128	21	107	112
16	" "	136	18	118	118
17	Diabetic.	159	18	141	146
18	" nephritic (non-protein N 105) receiving insulin.	101	18	83	83
19	Nephritic, terminal (non-protein N 143).	137	25	112	115
20	Diabetic (sugar before insulin 550) receiving insulin.	224	24	200	199
21a	Cardiac hypertension (non-protein N 95); completely digitalized, blood glycolyzed 4 hrs.	47	41	6	24
21b	Fresh sample July 19, 1928.	106	51	55	68
21c	" " (non-protein N 110) July 20, 1928.	130	47	83	92
21d	Fresh sample (non-protein N 207) July 22, 1928.	116	48	68	86
21e	Sample in ice box overnight; July 23, 1928, patient died.	123	42	81	89
22	Terminal uremia, sample on ice 24 hrs.	38	36	2	0
23	Diabetic.	288	15	273	278
24	" "	238	22	216	212

* F. W. = Folin-Wu filtrate, F.W.Y. = Folin-Wu filtrate after yeast fermentation. Sugar determinations were carried out on the filtrates in duplicate with the Shaffer-Hartmann reagent modified by Somogyi.

TABLE I—*Concluded.*

Sample No. (1)	Description of blood. (2)	Sugar, F.W.* (3)	Reducing non-sugar, F.W.Y.* (4)	True sugar, (3)-(4). (5)	Sugar, Hg(NO ₃) ₂ -BaCO ₃ . (6)
		<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
25a	Cardiac decompensation before digitalis.	117	21	96	96
25b	Completely digitalized (28 cc. digitalis for 6 days).	118	20	98	95
26a	Cardiac decompensation; not digitalized.	100	21	79	81
26b	Completely digitalized (25 cc. digitalis for 5 days).	128	21	107	107
27a	Cardiac decompensation; not digitalized.	123	19	104	101
27b	Completely digitalized (30 cc. digitalis for 5 days).	107	20	87	90
28	"Malignant hypertension" with uremia (non-protein N 250); died 2 hrs. after sample.	354	54	300	315
	Average (Samples 21 and 28 omitted).			103	103.6

substances of the blood and causing too high values by the mercury method. That this is not the explanation is shown in the results on Samples 25 to 27, determined both before and after digitalis treatment. Here the fermentation and mercury procedures check in a normal way. The blood from a case of terminal uremia, Sample 22, contrasts strikingly with the blood of Sample 21. The mercury precipitation removed all reducing non-sugars from the glycolyzed blood and showed a sugar value of 0 (Somogyi reagent), which was quite in agreement with the true sugar content by fermentation. It appears from such findings that in some cases of retention fairly large quantities of reducing (or iodine-consuming) non-fermentable substances, not precipitable by mercury, are present in the blood. This point is being further investigated.

The data of Table II give results for the sugar and reducing non-sugars in blood of several animal species. The reducing non-sugars, on the average, are lower for pig blood than any other blood examined, and yet the sugar content of pig blood, determined

TABLE II.
Animal Blood.

(1) Sample No.	(2) Description of blood.	(3) Sugar, F.W.* mg. per cent	(4) Reducing non- sugar, F.W.Y.* mg. per cent	(5) True sugar, (3)-(4). mg. per cent	(6) Sugar, Hg(NO) ₂ + BaCO ₃ . mg. per cent
1	Beef blood.	86	24	62	62
2	" "	88	25	63	65
3	" " same as Sample 2, in ice box 24 hrs.	88	22	66	62
4	Beef blood same as Sample 2, in ice box 48 hrs.	81	23	58	58
5	Rabbit blood.	143	26	117	114
6	Cat blood after ether anesthesia.	242	22	220	215
7	Pig "	72	16	56	60
8	" "	70	17	53	58
9	Same as Sample 8, in ice box 48 hrs.	68	16	52	58
10	Pig blood.	77	15	62	67
11	" "	71	18	53	54
12	" "	71	14	57	63
13	" "	65	17	48	51
14	" "	72	15	57	58
15	" " in ice box 24 hrs.	47	20	27	40
16	" " " " " 24 "	49	17	32	44
17	" "	77	13	64	68
18	" "	89	22	67	72
19	Same as Sample 18, in ice box 11 days.	69	20	49	55
20	Sheep blood.	71	22	49	54
21	" "	69	19	50	49
22	" "	59	19	40	46
23	" "	58	16	42	39
24	" "	51	17	34	35

* F.W. = Folin-Wu filtrate, F.W.Y. = Folin-Wu filtrate after yeast fermentation. Sugar determinations were carried out on the filtrates in duplicate with the Shaffer-Hartmann reagent modified by Somogyi.

by the mercury method, is generally several mg. per cent higher than the true sugar by fermentation. Mercury filtrates of some fresh pig bloods absorb a little iodine in acid solution, but this if included in the sugar results would be equivalent to no more than 2 to 3 mg. per cent of sugar in the Shaffer-Hartmann procedure. Mercury filtrates prepared from pig bloods kept in the ice box for some days contain much less iodine-absorbing materials but they

TABLE III.
Mercuric Sulfate Filtrates.

Sample No.	Blood.	Sugar by fermentation.	Sugar, mercuric sulfate filtrates.	Sugar, mercuric nitrate filtrates.
		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	Beef.	158	160*	
2	"	213	215	
3	"	253	252	
4	Pig.	56	54*	
5	"	46	51	51
6	Human, normal.	75	76	
7	" cardiac decompensation.	107	108	
8	" " "	87	84	
9	" " "	98	97	
10	Hypertension with uremia (non-protein N 185).	81	90	89
11	"Malignant hypertension" with uremia (non-protein N 250).	300	310	315
12	Catarrhal jaundice.	114	114*	
13	Cardiac decompensation.	105	108*	
14	Congenital cystic kidneys (non-protein N 168).	79	80*	

* Mercury removed with zinc dust; in other cases removed with hydrogen sulfide.

still show about the same amount of sugar in excess of the fermentation methods as do filtrates of fresh blood. These facts seem to point to the conclusion that pig blood is unique among the animal bloods examined (with the possible exception of sheep blood) in containing appreciable quantities of an unknown reducing substance which is not precipitated by mercury and which is not destroyed by keeping the blood for a long time at low temperature.

It is interesting to speculate concerning the nature of these iodine-absorbing substances present in mercury filtrates of some pig bloods. It is improbable that thioneine and glutathione, both of which are present in pig blood (18), contribute to these substances because both compounds are precipitated by mercury. They resemble the iodine-consuming substances found in Folin-Wu filtrates cited by Somogyi (2) in that both disappear from blood on standing.

The data of Table III show that mercuric sulfate-barium carbonate precipitation of blood yields filtrates which give true sugar values as judged by those of yeast fermentation. In a few cases, Samples 5, 10, and 11, determinations were made on mercuric nitrate filtrates also. The results are identical within the limits of experimental error. Sample 11 shows a result 5 mg. per cent higher by the nitrate method than by the sulfate. This is probably due to salt error in the nitrate filtrates. Samples 10 and 11 are the same as Samples 21 and 28 of Table I and show the same exception to the sulfate method as to the nitrate.

The writers feel that the mercuric sulfate-barium carbonate technique, with zinc and the modified Shaffer-Hartmann sugar reagent, constitutes a procedure much superior to the nitrate method for the direct determination of true blood sugar. To summarize, it is much less laborious, very much faster, and probably a bit more accurate than the nitrate method because the salt error is ruled out. It is being found very satisfactory in these laboratories for the treatment of urine, hydrolyzed tissues, etc., in that no electrolytes which are not removed are introduced and there is little or no salt error in the sugar determinations. The case is quite the opposite with mercuric nitrate precipitation.

The writers are indebted to Mr. Earle Adler for technical assistance in carrying out many of the analyses. Mr. A. H. Dowdy also has aided in some of the determinations. To Dr. D. P. Barr of the Department of Medicine we wish to express our appreciation for his interest in the work and a generous supply of pathological bloods from Barnes Hospital. Dr. M. Somogyi has kindly given us many helpful suggestions and supplied some of the bloods used. To Professor Shaffer we owe much for valuable suggestions and criticism.

SUMMARY.

A study has been made of the preparation of blood filtrates for sugar analysis by precipitation with mercuric nitrate followed by neutralization with barium carbonate. The neutralization proceeds automatically and consistently to a pH of about 6.3. Filtrates prepared from human blood by this procedure, both normal and diabetic, yield true sugar values as judged by those obtained with Somogyi's yeast fermentation method. A case of cardiac hypertension with high nitrogen retention proved an exception to the usual close agreement. Cases of uremia and diabetes-nephritis gave true sugar values on mercury filtrates.

Some pig bloods contain a non-fermentable reducing substance (or substances) not completely removed by mercuric nitrate-barium carbonate treatment. Pig bloods also often contain some iodine-absorbing substance which apparently is neither glutathione nor thionine.

A mercuric sulfate-barium carbonate-zinc technique is introduced for the preparation of filtrates for sugar analysis. It is simple, rapid, and probably a bit more accurate than the mercuric nitrate-barium carbonate method because the salt error is ruled out. It is especially recommended for the preparation of filtrates of urine, hydrolyzed tissues, etc., as well as blood, because *no electrolytes from the precipitating agent* remain in the filtrates.

Addendum.—Recently it has been found that Folin-Wu filtrates may be very simply treated by the mercuric sulfate-barium carbonate-zinc technique to give both true sugar and reducing non-sugar values.

Add 1 drop of 1:1 H_2SO_4 to 20 to 25 cc. of Folin-Wu filtrate and then about 0.5 gm. of solid mercuric sulfate, followed by vigorous shaking for 15 to 20 seconds. Considerable mercuric sulfate is converted into the basic sulfate and does not dissolve. This in no way interferes with the determination. Add 1 to 2 gm. of precipitated BaCO_3 . Shake until most of the CO_2 has escaped, then stopper the flask, and shake until no further pressure develops and the mixture is neutral to litmus. If necessary add a little more BaCO_3 . Filter, add 1 drop of saturated Na_2SO_4 solution, a pinch of Zn dust, shake, and filter. The sugar is determined in the filtrate by the modified Shaffer-Hartmann reagent cited above, which is unaffected by the peroxide contained in the Zn-treated filtrates. The sugar value obtained on the original Folin-Wu filtrate minus that on the mercury-treated filtrate represents the reducing non-sugar. This procedure is especially recommended for use when it is necessary to prepare Folin-Wu filtrates for a number of different determinations and it is desirable to estimate quickly and

accurately true sugar and reducing non-sugar. It is also much less expensive than the direct treatment of blood with the mercuric sulfate solution, and some workers may prefer to prepare Folin-Wu filtrates first and then treat them as outlined. Filtrates prepared in this way contain some nitrogen but the reducing non-sugars are completely removed. Professor Shaffer has observed that mercuric acetate may be satisfactorily substituted for mercuric sulfate in the treatment of Folin-Wu filtrates. Urine and hydrolyzed tissues cannot be effectively precipitated with *solid* mercuric sulfate.

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THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES.

V. ENZYME HYDROLYSIS OF DIPEPTIDES.

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Previous investigations in this laboratory have indicated that the resistance of peptides to chemical hydrolytic agents is conditioned by more than one factor. Two factors have been disclosed with certainty. The first is the intrinsic constants of the groups involved in the peptide linkage.¹ The second, which may be termed steric, is the condition of that carbon atom which carries the amino group involved in the peptide linkage; peptides in which this carbon atom does not carry a mobile hydrogen atom (tertiary carbon atoms) show great resistance.²

The question arises whether the same factors are operative in the case of enzymatic hydrolysis, or whether in this case the number of factors is still more manifold. Abderhalden³ and his associates have reported many instances in which a peptide containing in its chain an amino acid enantiomorphous to that occurring naturally is not hydrolyzed by enzymes; these results bring out a factor very important in enzymatic action, but non-existent in the case of hydrolysis by chemical agents. Northrop and Simms⁴ have recently pointed out the significance of the dissociation con-

¹ Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 445 (1924).

² Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **82**, 167 (1929).

³ Abderhalden, E., *Lehrbuch der physiologischen Chemie*, Berlin and Vienna, 5th edition, **2**, 293-301 (1923). Abderhalden, E., and Schapiro, N., *Fermentforschung*, **9**, 234 (1927).

⁴ Northrop, J. H., and Simms, H. S., *J. Gen. Physiol.*, **12**, 313 (1928-29).

stants of one of the free functional groups of the substrate and of that of the enzyme. Thus two additional factors have been shown to exert an influence on the rates of enzymatic hydrolysis of

TABLE I.
Hydrolysis Constants of Peptides.

Peptide.	Concentration.	$k' = k \cdot 10^3$	
		Previously reported.	Present investigation.
Simple peptides.			
Glycyl-glycine.....	0.025	74	29
“	0.050	47	
“	0.100	26	
“	0.500	4	
“ + 2 equivalents glycine.....	0.050	22	
Glycyl- <i>d,l</i> -alanine.....	0.100	87	110
“	0.200	40	
Glycyl-dextro-valine.....	0.100	22	
Glycyl-levo-valine.....	0.100	0	
Glycyl-levo-leucine.....	0.100	33	
<i>d,l</i> -Alanyl-glycine.....	0.100	150	
“	0.200	60	
Levo-alanyl-glycine.....	0.100	0	
<i>d,l</i> -Alanyl- <i>d,l</i> -alanine.....	0.100	8	
Dextro-alanyl-dextro-alanine.....	0.100	150	
Levo-alanyl-dextro-alanine.....	0.100	0	55*
Glycyl- <i>d,l</i> -phenylaminoacetic acid.....	0.100		
“ “	0.200		
Glycyl-levo-phenylaminoacetic acid.....	0.100	0	
Glycyl- <i>d,l</i> -phenylalanine.....	0.100	19	
“	0.200	10*	
Peptides with a tertiary α -carbon atom.			
Glycyl- α -aminoisobutyric acid.....	0.100		0
Glycyl-dextro-isovaline.....	0.100	0	
Glycyl- <i>d,l</i> -phenylmethylaminoacetic acid....	0.100		0

* Estimated.

peptides. In fact, in the light of the work of Northrop and Simms, some of the older conclusions regarding the reactivity of peptides may require revision.

The results of investigations on enzyme hydrolysis published in a previous paper⁵ and some of the results presented here were obtained prior to the publication of the paper of Northrop and Simms. The conditions in our experiments were not identical to those chosen by these authors. Nevertheless, our conditions are satisfactory for our purpose for the reason that the hydrogen ion concentration in the hydrolyses is that at which Northrop and Simms observed the maximum hydrolysis for peptides composed of monoaminomonocarboxylic acids. Furthermore, the concentrations of substrate and enzyme were so chosen that the course of the reactions followed the mass law in its monomolecular form. The hydrolysis constants under these conditions are directly proportional to the concentration of enzyme and inversely proportional to the concentration of substrate. Finally, under these conditions hydrolysis proceeded to completion. Hence the differences in the rates of hydrolysis of individual peptides tested under identical conditions may be attributed to the intrinsic properties of the peptides and not to the external conditions.

The results of our experiments published in the previous paper and those reported here are summarized in Table I. The following conclusions are drawn from these results.

1. The peptides containing one component enantiomorphous to the natural amino acids are not hydrolyzed by erepsin. This conclusion, which is in harmony with the observations of Abderhalden and his coworkers, is readily explained on the basis of a relationship between the configurations of substrate and of enzyme known to exist in the case of other enzymes.

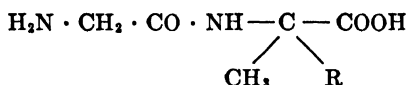
2. Dipeptides containing one *d,l*-amino acid and one naturally occurring amino acid are hydrolyzed to the extent of 50 per cent. Abderhalden⁶ and his coworkers report cases in which such dipeptides were not hydrolyzed by erepsin; these experiments should be repeated.

3. Dipeptides in which the free carboxyl group and the amino

⁵ Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **81**, 221 (1929).

⁶ See Abderhalden, E., and Köppel, W., *Fermentforschung*, **9**, 445 (1928). Abderhalden, E., and Rossner, E., *Fermentforschung*, **9**, 497 (1928).

group involved in the peptide linkage are attached to a tertiary carbon atom



were not hydrolyzed by erepsin in the time intervals of our experiments.

4. In equivalent concentrations the following relationships exist: (a) Glycyl-glycine ($k' = 26$) and glycyl-dextro-valine ($k' = 22$) hydrolyze at approximately the same rate. (b) Glycyl-levo-leucine ($k' = 33$) hydrolyzes at a somewhat higher rate. (c) Dextro-alanyl-dextro-alanine ($k' = 150$) hydrolyzes at the highest rate. (d) Dextro-alanyl-glycine (in *d,l*-alanyl glycine; $k' = 60$) shows a higher rate of hydrolysis than glycyl-dextro-alanine (in glycyl-*d,l*-alanine; $k' = 40$). (e) Finally, an interesting comparison is furnished by two peptides which differ from each other in the distance between the phenyl and amino groups. Thus the rates of hydrolysis of glycyl-dextro-phenylaminoacetic acid⁷ (in glycyl-*d,l*-phenylaminoacetic acid; $k' = 55$) and of glycyl-levo-phenylalanine (in glycyl-*d,l*-phenylalanine; $k' = 10$) are in the ratio of 55 to 10.

It was mentioned earlier in this article that in the action of chemical agents the rate of hydrolysis by acid is conditioned principally by the intrinsic constants of the groups involved in the peptide linkage. From the observations presented in Table I it seems that in enzymatic hydrolysis the chemical structure of the substrate plays an equally important part. Thus it would be difficult to explain the great difference between the rates of hydrolysis of glycyl-glycine and of *d,l*-alanyl-glycine solely on the basis of the differences in the dissociation constants either of the groups involved in the peptide linkage, or of the free functional groups. On the other hand, the marked difference in the

⁷ Phenylaminoacetic acid has not been found to occur as a constituent of proteins. Other peptides containing amino acids which have not been isolated from natural products have been shown by Waldschmidt-Leitz and coworkers [Waldschmidt-Leitz, E., Schäffner, A., Schlatter, H., and Klein, W., *Ber. chem. Ges.*, **61**, 299 (1928)] to be split by the action of erepsin.

rates of hydrolysis of glycyl-*d,l*-phenylaminoacetic acid and of glycyl-*d,l*-phenylalanine is undoubtedly due in part to the differences in the dissociation constants of the groups involved in the peptide linkage. This peculiarity in the action of enzymes may be of great advantage in their application to the further study of the structure of proteins.

We wish to thank Dr. Masao Osaki also Mr. R. E. Marker for their assistance in the preparation of the peptides used in these experiments.

EXPERIMENTAL.

Preparation of Peptides.

The analyses of the peptides used in these experiments are summarized in Table II.

TABLE II.

Analyses of Peptides.

The results are expressed in per cent.

	Sam- ple No.	Calculated.			Found.			
		C	H	N	C	H	N	Mois- ture.
Glycyl-glycine.....	2501	36.34	6.11	21.21	36.63	6.27	21.10	2.26
Glycyl- α -aminoisobutyric acid.....	2680	44.97	7.56	17.50	44.77	7.29	17.29	1.50
Glycyl- <i>d,l</i> -phenylamino- acetic acid.....	2685	57.66	5.81	13.46	57.35	6.01	13.03	0.46
Glycyl-levo-phenylamino- acetic acid.....	2721	57.66	5.81	13.46	57.94	5.77	13.16	0.90
Glycyl- <i>d,l</i> -phenylalanine..	2681	59.43	6.35	12.61	59.12	6.49	12.48	1.06
Glycyl- <i>d,l</i> -phenylmethyl- aminoacetic acid.....	2683	59.43	6.35	12.61	59.60	6.48	12.39	0.87

Glycyl-Glycine.—The same sample was used as in the preceding publication.⁵

Glycyl- α -Aminoisobutyric Acid.—Chloroacetyl- α -aminoisobutyric acid. A solution of 42 gm. (0.4 mol) of α -aminoisobutyric acid in 200 cc. of 2.0 *N* sodium hydroxide (0.4 mol) was treated alternately with 91 gm. (0.8 mol) of chloroacetyl chloride and 200 cc. of 5.0 *N* sodium hydroxide (1 mol) with cooling in ice and continuous shaking, the reagents being added in equivalent proportions. The

chloroacetyl compound precipitated on addition of 140 cc. of 5.0 N hydrochloric acid (0.7 mol). It was washed with ice water and dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It weighed 53.5 gm.

Glycyl- α -aminoisobutyric acid. 53.5 gm. of crude chloroacetyl- α -aminoisobutyric acid were allowed to stand 3 days at room temperature with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The solution was then diluted with water, shaken with norit, filtered, and concentrated to dryness under reduced pressure. The mixture of dipeptide and ammonium chloride weighed 72 gm. It was dissolved in 66 gm. of hot water in the presence of some norit, and 500 cc. of hot absolute alcohol were added to the filtrate. Crystallization of the dipeptide, which set in immediately, was completed by cooling in ice for 24 hours. The crystals were washed with 60 per cent (by weight) alcohol, then with absolute alcohol. Yield 21 gm. A sample of 6 gm. was further purified. It was dissolved in 14 cc. of hot water and norit was added; 200 cc. of absolute alcohol were added to the filtrate. The crystals were filtered off after standing overnight in ice and were washed with absolute alcohol, then with ether.

3.950 mg. substance: 6.485 mg. CO₂ and 2.570 mg. H₂O.

0.1000 gm. " : 12.35 cc. 0.1 N HCl (Kjeldahl).

C₈H₁₃O₃N₂ (160.12). Calculated. C 44.97, H 7.56, N 17.50.

Found. " 44.77, " 7.29, " 17.29.

Glycyl-Levo-Phenylaminoacetic Acid.—Levo-phenylaminoacetic acid. *d,l*-Phenylaminoacetic acid was resolved by means of dextro-camphorsulfonic acid according to the directions of Ingersoll.⁸ The purified levo acid gave the following analysis.

0.1003 gm. substance: 0.2334 gm. CO₂ and 0.0538 gm. H₂O.

0.1000 " " : 6.65 cc. 0.1 N HCl (Kjeldahl).

C₈H₉O₂N (151.08). Calculated. C 63.54, H 6.00, N 9.27.

Found. " 63.45, " 6.00, " 9.31.

Moisture 0.22 per cent.

It gave the following rotations, all in 0.25 M solution (corrected for moisture).

⁸ Ingersoll, A. W., *J. Am. Chem. Soc.*, **47**, 1170 (1925).

$$[\alpha]_D^{20} = \frac{-2.70^\circ \times 100}{4 \times 0.604} = -111.7^\circ \text{ (in water);}$$

$$[\alpha]_D^{20} = \frac{-3.54^\circ \times 100}{4 \times 0.604} = -146.5^\circ \text{ (with 1 equivalent of HCl);}$$

$$[\alpha]_D^{20} = \frac{-2.81^\circ \times 100}{4 \times 0.604} = -116.3^\circ \text{ (with 1 equivalent of NaOH).}$$

Fischer and Weichhold⁹ give $[\alpha]_D^{20} = +112.6 (\pm 3^\circ)$ for a 0.4795 per cent aqueous solution of dextro-phenylaminoacetic acid, and $[\alpha]_D^{20} = -157.87^\circ (\pm 0.5^\circ)$ for a solution of 0.5387 gm. of levo-phenylaminoacetic acid in 4.85 cc. of 1.0 N hydrochloric acid and 2.17 cc. of water.

Bromoacetyl-levo-phenylaminoacetic acid. A solution of 15.2 gm. (0.1 mol) of levo-phenylaminoacetic acid in 50 cc. of 2.0 N sodium hydroxide (0.1 mol) was treated alternately with 21 gm. of bromoacetyl bromide (0.1 mol) and 60 cc. of 2.0 N sodium hydroxide (0.12 mol) with cooling in ice and continuous shaking, the reagents being added in equivalent proportions. Addition of 30 cc. of 5.0 N hydrochloric acid (0.15 mol) yielded a pasty precipitate, which became solid when the mixture was allowed to stand for several hours at room temperature. The bromoacetyl compound was washed with ice water, and was dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It weighed 20 gm.

Glycyl-levo-phenylaminoacetic acid. 20 gm. of crude bromoacetyl-levo-phenylaminoacetic acid were allowed to stand 3 days at room temperature with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The solution was then diluted with water, shaken with norit, filtered, and concentrated to dryness under reduced pressure. The residue of dipeptide and ammonium bromide was dissolved in 159 gm. of boiling water in the presence of some norit, and 700 cc. of absolute alcohol were added to the filtrate. The mixture was cooled for 2 days in ice. 8.1 gm. of crude dipeptide were thus obtained. An identical yield was obtained in a second preparation. For purification, 5 gm. of this material were digested with 70 cc. of boiling water (some remained undissolved, probably levo-phenylaminoacetic acid). Norit was

⁹ Fischer, E., and Weichhold, O., *Ber. chem. Ges.*, **41**, 1286 (1908).

then added, the solution was filtered, and the dipeptide was precipitated with 450 cc. of absolute alcohol. It was washed with absolute alcohol, then with ether.¹⁰ Yield 3.75 gm.

3.720 mg. substance: 7.905 mg. CO₂ and 1.920 mg. H₂O.

0.1000 gm. " : 9.40 cc. 0.1 N HCl (Kjeldahl).

C₁₀H₁₂O₃N₂ (208.12). Calculated. C 57.66, H 5.81, N 13.46.

Found. " 57.94, " 5.77, " 13.16.

$$[\alpha]_D^{25} = \frac{-7.24^\circ \times 100}{2 \times 2.082} = -173.9^\circ \text{ (in water);}$$

0.1041 gm. (dry) in 5.00 cc. of solution (0.1 M).

Glycyl-d,l-Phenylaminoacetic Acid.—Bromoacetyl-*d,l*-phenylaminoacetic acid. This compound was prepared from *d,l*-phenylaminoacetic acid by a procedure identical to that used for the levo isomer. It has previously been obtained by Petrescu.¹¹

Glycyl-*d,l*-phenylaminoacetic acid. This derivative was prepared from 20 gm. of crude bromoacetyl-*d,l*-phenylaminoacetic acid. The same procedure was used as in the case of the levo isomer. The compound has previously been obtained by Petrescu.¹¹

The mixture of dipeptide and ammonium bromide was dissolved in 203 gm. of boiling water in the presence of some norit, and 700 cc. of absolute alcohol were added to the filtrate. The mixture was cooled in ice for 2 days. The slight, cloudy precipitate which had formed was removed by filtration; crystallization then took place overnight. 6.75 gm. of crude dipeptide were thus obtained. An identical yield was found in a second preparation. For purification, 15.5 gm. of this material were digested with boiling water (some remained undissolved, probably *d,l*-phenylaminoacetic acid). The filtrate was concentrated to a small volume under reduced pressure and treated with norit. On addition of an excess of absolute alcohol to the filtrate, 8.74 gm. of peptide precipitated.¹⁰

¹⁰ Judging from the method of preparation and from the results of analysis, it seems probable that this substance was contaminated with phenylaminoacetic acid.

¹¹ Petrescu, L., *Bull. soc. chim. Romania*, **1**, 56 (1919); abstracted in *Chem. Abst.*, **15**, 69 (1921).

3.100 mg. substance: 6.520 mg. CO₂ and 1.685 mg. H₂O.

0.1000 gm. " : 9.31 cc. 0.1 N HCl (Kjeldahl).

C₁₀H₁₂O₃N₂ (208.12). Calculated. C 57.66, H 5.81, N 13.46.

Found. " 57.35, " 6.01, " 13.03.

Glycyl-d,l-Phenylalanine.—This peptide was prepared according to the directions given in the literature.¹²

Glycyl-d,l-Phenylmethylaminoacetic Acid.—Chloroacetyl-*d,l*-phenylmethylaminoacetic acid. The preparation was carried out in a similar way to that of chloroacetyl- α -aminoisobutyric acid. The proportions of the reagents were the following: 33 gm. (0.2 mol) of *d,l*-phenylmethylaminoacetic acid dissolved in 100 cc. (0.2 mol) of 2.0 N sodium hydroxide, 45 gm. (0.4 mol) of chloroacetyl chloride, and 100 cc. (0.5 mol) of 5.0 N sodium hydroxide; finally, 72 cc. of 5.0 N hydrochloric acid for precipitating the chloroacetyl compound, which weighed 48.8 gm.

Glycyl-*d,l*-phenylmethylaminoacetic acid. The peptide was prepared and purified by a procedure similar to that used for glycyl- α -aminoisobutyric acid. The mixture of peptide and ammonium chloride from 48.8 gm. of crude chloroacetyl-*d,l*-phenylmethylaminoacetic acid was dissolved in 562 gm. of boiling water in the presence of some norit, and the crude dipeptide was precipitated with 1500 cc. of hot absolute alcohol. Yield 26.1 gm. It was dissolved in 525 gm. of boiling water and norit was added; 1500 cc. of hot absolute alcohol were added to the filtrate. Yield 19.3 gm.

3.685 mg. substance: 8.055 mg. CO₂ and 2.135 mg. H₂O.

0.1000 gm. " : 8.85 cc. 0.1 N HCl (Kjeldahl).

C₁₁H₁₄O₃N₂ (222.13). Calculated. C 59.43, H 6.35, N 12.61.

Found. " 59.60, " 6.48, " 12.39.

Preparation of Enzyme Solution.

The erepsin solution used in this series of experiments was prepared by exactly the same method as that used in the previous series. The two solutions were of nearly the same strength, a fact which is shown by the values of $k \cdot 10^3$ for glycyl-glycine (0.100 M) in the two series, viz. 26 and 29.

¹² Leuchs, H., and Suzuki, U., *Ber. chem. Ges.*, **37**, 3313 (1904).

TABLE III.
*Hydrolysis of Peptides by Erepsin.**

Solutions contained 0.005 mol of peptide, 0.3 equivalent of alkali, and 5.00 cc. of enzyme solution. Total volume, 50.0 cc; samples for analysis, 5.00 cc. Titration with 0.2 N alkali.

Control.†			Glycyl-glycine.			Glycyl- α -amino- isobutyric acid.			Glycyl- β -L-phenyl- aminoacetic acid.			Glycyl- β -L-phenyl- alanine.			Glycyl- β -L-phenyl- methylaminoacetic acid.		
<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$
<i>hrs.</i>	cc.		<i>hrs.</i>	cc.		<i>hrs.</i>	cc.		<i>hrs.</i>	cc.		<i>hrs.</i>	cc.		<i>hrs.</i>	cc.	
0	1.74		0	1.78		0	1.78		0	1.76		0	1.75		0	1.78	
0.75	1.75	0	1.0	1.90	(22)	0.75	1.77	0	0.75	1.92	(85)	0.75	1.79	19	0.75	1.78	0
1.5	1.75	0	1.5	1.99	25	1.50	1.78	0	1.5	2.12	10 ₂	1.5	1.83	19	1.5	1.79	0
4.0	1.74	0	2.5	2.15	28	4.0	1.78	0	2.25	2.30	11 ₂	2.5	1.86	16	2.25	1.80	0
9.0	1.75	0	4.0	2.34	28	9.0	1.79	0	3.0	2.42	11 ₁	4.0	1.94	18	3.0	1.79	0
24.0	1.75	0	6.0	2.62	30	24.0	1.80	0	4.0	2.60	12 ₄	6.0	2.05	20	9.0	1.80	0
51.0	1.76	0	9.0	2.95	30	51.0	1.89		9.0	2.90		9.0	2.15	19	25.0	1.83	0
			24.0	3.84	31				25.0	2.92		24.0	2.83		49.0	1.85	
			51.0	4.23					49.0	2.94		51.0	3.06				
Average...		0			29			0			11 ₂			19			0

* Values of $k \cdot 10^3$ in parentheses have not been included in calculating average values; inferior numbers are used to indicate figures which are not significant.

† Solution of 0.005 mol of peptide and 0.3 equivalent of alkali; no erepsin.

Procedure in Hydrolysis Experiments.

The procedure is exactly the same as that described in the previous publication. The data are recorded in Table III. Control experiments with 0.100 M glycyl-glycine and erepsin were run for each series of hydrolyses; the hydrolysis constants of these controls were identical within the limits of error.

Hydrolysis of Glycyl-d,l-Phenylalanine.

A solution of 0.005 mol of peptide, 0.3 equivalent of alkali, and 5.0 cc. of enzyme solution was made up to a volume of 50.0 cc. A sample of 2.50 cc. required (formol titration) 1.77 cc. of 0.2 N alkali. A sample of 10.0 cc. was neutralized with the theoretical quantity of hydrochloric acid, diluted to 25.0 cc., and filtered. The rotation in a 4 dm. tube was $\alpha_D^{25} = 0^\circ$.

The original solution was then allowed to stand 24 hours at 37° . A sample of 5.00 cc. required (formol titration) 2.79 cc. of 0.2 N alkali. The theoretical value, assuming complete hydrolysis of glycyl-levo-phenylalanine and no hydrolysis of glycyl-dextro-phenylalanine, is 3.02 cc. of 0.2 N alkali. A sample of 10.0 cc. was neutralized with the theoretical quantity of hydrochloric acid, diluted to 25.0 cc., and filtered. The rotation in a 4 dm. tube was $\alpha_D^{26} = -0.89^\circ$.

Assuming complete hydrolysis of the levo compound and no hydrolysis of the dextro compound, the 25.0 cc. solution used for measuring the rotation should contain 0.1111 gm. of glycyl-dextro-phenylalanine and 0.0826 gm. of levo-phenylalanine. From the values of the rotations of these compounds in aqueous solution,¹³ (levo-phenylalanine, $[\alpha]_D^{20} = -35.14^\circ (\pm 0.5)$; glycyl-dextro-phenylalanine, $[\alpha]_D^{20} = -42.0^\circ (\pm 0.5)$) the calculated value of the rotation of this mixture in a 4 dm. tube is $\alpha_D^{25} = -1.21^\circ$.

Hydrolysis of Glycyl-d,l-Phenylaminoacetic Acid.

A solution of 0.005 mol of peptide, 0.3 equivalent of alkali, and 5.0 cc. of enzyme solution was made up to a volume of 50.0 cc. A sample of 5.0 cc. required (formol titration) 1.76 cc. of 0.2 N alkali. A sample of 10.0 cc. was neutralized with the theoretical

¹³ See Fischer, E., and Schoeller, W., *Ann. Chem.*, **357**, 1 (1907).

quantity of hydrochloric acid, diluted to 25.0 cc., and filtered. The rotation in a 4 dm. tube was $\alpha_D^{25} = 0^\circ$.

The original solution was then allowed to stand 15 hours at 37° . A sample of 5.00 cc. required (formol titration) 2.92 cc. of 0.2 N alkali. The theoretical value, assuming complete hydrolysis of glycyl-dextro-phenylaminoacetic acid and no hydrolysis of glycyl-levo-phenylaminoacetic acid, is 3.01 cc. of 0.2 N alkali. A sample of 10.0 cc. was neutralized with the theoretical quantity of hydrochloric acid, diluted to 25.0 cc., and filtered. The rotation in a 4 dm. tube was $\alpha_D^{25} = -1.39^\circ$.

Assuming complete hydrolysis of the dextro compound and no hydrolysis of the levo compound, the 25.0 cc. solution used for measuring the rotation should contain 0.1041 gm. of glycyl-levo-phenylaminoacetic acid and 0.0756 gm. of dextro-phenylaminoacetic acid. From the values of the rotations of these compounds in aqueous solution (dextro-phenylaminoacetic acid, $[\alpha]_D^{25} = +112^\circ$; glycyl-levo-phenylaminoacetic acid, $[\alpha]_D^{25} = -174^\circ$) the calculated value of the rotation of this mixture in a 4 dm. tube is $\alpha_D^{25} = -1.55^\circ$.

THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES.

VI. HYDROLYSIS OF DIPEPTIDES BY ALKALI.

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In connection with an investigation of the action of alkali on proteins,¹ undertaken for the purpose of obtaining data which might aid in establishing the structure of these complex molecules, the properties of simple type compounds believed to occur in the protein molecule are being studied in this laboratory. The results obtained on the racemization of peptides by alkali,² on the hydrolysis of peptides by hydrochloric acid,³ and by erepsin,⁴ on the racemization of ketopiperazines by alkali,² and on the hydrolysis of N-methylketopiperazines by alkali,⁵ have been reported in other papers.

The present investigation deals with the hydrolysis of dipeptides by alkali. Other workers, notably Abderhalden⁶ and his col-

¹ Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **74**, 715 (1927); **78**, 145 (1928); **82**, 171 (1929).

² Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, **63**, 661 (1925); *J. Gen. Physiol.*, **8**, 183 (1925); *J. Biol. Chem.*, **68**, 277 (1926); **70**, 219 (1926). Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 299 (1928).

³ Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 445 (1924).

⁴ Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **62**, 711 (1924-25). Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **70**, 253 (1926). Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **81**, 221 (1929). Levene, P. A., Steiger, R. E., and Bass, L. W., *J. Biol. Chem.*, **82**, 155 (1929).

⁵ Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **81**, 697 (1929).

⁶ Abderhalden, E., and coworkers, many papers in *Fermentforschung* and *Z. physiol. Chem.* since 1927.

TABLE I.
*Hydrolysis Constants of Peptides.**

Glycyl-glycine.			Glycyl-d, l- alanine.			d, l-Alanyl- d, l-alanine.			Glycyl- α -amino- isobutyric acid.			Glycyl-d, l,- phenylamino- acetic acid.			Glycyl-d, l- phenylalanine.			Glycyl-d, l- phenylmethyl- aminoacetic acid.		
<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$	<i>t</i>	<i>V</i>	$k \cdot 10^3$
days	cc.		days	cc.		days	cc.		days	cc.		days	cc.		days	cc.		days	cc.	
0	2.35		0	2.45		0	2.35		0	2.37		0	2.25		0	2.35		0	2.38	
1	3.15 (168)		1	2.59 (25)		1	2.35 (0)		1	2.35	0	2	2.46	19	4	2.51	7.1	1	2.30	0
2	3.64 158		2	2.83	36	2	2.45	8.9	2	2.40	0	4	2.73	23	7	2.66	8.2	2	2.33	0
3	3.96 150		3	2.90	29	3	2.43 (4.7)		4	2.41	0	7	3.06	24	10	2.80	8.6	4	2.35	0
4	4.24 153		7	3.49	33	7	2.60	6.6	7	2.41	0	10	3.22	21	14	2.89	7.6	7	2.34	0
7	4.62 148		10	3.79	33	10	2.77	8.0	10	2.37	0	14	3.60	24	21	3.13	7.8	10	2.37	0
10	4.80 (170)		14	4.00	30	14	2.85	6.9	14	2.40	0	21	3.89	22				14	2.35	0
14	4.80								21	2.42	0							21	2.35	0
Average.	152																			
					32			7.6			0			22			7.9			0

* Values of $k \cdot 10^3$ in parentheses have not been included in calculating average values.

laborators, have studied the hydrolysis of peptides by alkali, but their results are not always comparable. Furthermore, their data were obtained under conditions differing from those adopted for our systematic investigations on proteins, peptides, and ketopiperazines.

The data on the hydrolysis at 25° of eight dipeptides are presented in Table I. From this table it is apparent that the hydrolysis constants of peptides vary widely according to their structures. In a previous paper³ it has been shown that in the case of acid hydrolysis the rates of hydrolysis are a function of the intrinsic constants of the groups involved in the peptide linkage. Apparently this relation does not hold for alkaline hydrolysis under the conditions which we have used. The hydrolysis solutions were at a pH of approximately 13.4, a hydrogen ion concentration beyond the range of the dissociation constants of the groups forming the peptide linkage. In this range it seems more probable that the dissociation constants of the enolic forms⁷ are the determining factor in hydrolysis.

From Table I it is seen that the rates of hydrolysis decrease in the following order: glycyl-glycine, glycyl-alanine and alanyl-glycine, glycyl-phenylaminoacetic acid, alanyl-alanine and glycyl-phenylalanine. Dipeptides in which the free carboxyl group and the amino group involved in the peptide linkage are attached to a tertiary carbon atom (glycyl- α -aminoisobutyric acid and glycyl-phenylmethylaminoacetic acid) exhibit great stability; similar stability was observed in the case of hydrolysis by erepsin.

EXPERIMENTAL.

Preparation of Peptides.—The samples of dipeptides used in this investigation are the same as those described previously:⁸ glycyl-glycine, No. 2501; glycyl-*d*, *l*-alanine, No. 2516; *d*, *l*-alanyl-glycine, No. 2502; *d*, *l*-alanyl-*d*, *l*-alanine, No. 2504; glycyl- α -aminoisobutyric acid, No. 2680; glycyl-*d*, *l*-phenylaminoacetic acid,

⁷ Cf. Levene, P. A., Bass, L. W., Rothen, A., and Steiger, R. E., *J. Biol. Chem.*, **81**, 687 (1929).

⁸ Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **81**, 221 (1929). Levene, P. A., Steiger, R. E., and Bass, L. W., *J. Biol. Chem.*, **82**, 155 (1929).

No. 2685; glycyl-*d,l*-phenylalanine, No. 2681; glycyl-*d,l*-phenyl-methylaminoacetic acid, No. 2683.

Procedure in Hydrolysis Experiments.—The following procedure was used for each peptide. The rates of hydrolysis were measured at 25°.

A sample of 0.005 mol (corrected for moisture) of peptide was weighed in a 50.0 cc. volumetric flask. The sample was dissolved in water, 25.0 cc. (5 equivalents) of 1.0 *N* sodium hydroxide were added, the volume was made up to 50.0 cc. with water, and the solution was thoroughly mixed. At definite time intervals, 5.00 cc. samples were withdrawn by means of pipettes. The sodium hydroxide in each sample was neutralized by adding 2.50 cc. of 1.0 *N* hydrochloric acid. The amino acid-peptide mixture was titrated with 0.2 *N* sodium hydroxide by Sørensen's formol titration method, thymolphthalein being used as indicator.

The rates of hydrolysis were calculated by the equation

$$kt = \log_{10} \frac{a}{a - x}$$

The results are given in Table I.

STUDIES ON RACEMIZATION.

VIII. THE ACTION OF ALKALI ON PROTEINS: RACEMIZATION AND HYDROLYSIS.

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When the investigation of the action of alkali on proteins^{1,2} was undertaken by us, the problem seemed very simple. It was stimulated by observations on the action of alkali on ketopiperazines on one hand, and on simple peptides on the other.³ The effect of 0.1 to 0.2 *N* alkali on peptides was minimal; in the case of ketopiperazines it produced rapid racemization to the extent of about 80 per cent in the course of 24 hours. Strong alkali (0.5 to 1.0 *N*) produced hydrolysis of di-, tri-, and tetrapeptides, and racemization of tri- and tetrapeptides which proceeded at a very slow rate; in the case of ketopiperazines it produced rapid hydrolysis⁴ to the corresponding dipeptides and very little racemization. Thus rapid hydrolysis with 1.0 *N* alkali and rapid racemization with 0.1 *N* alkali are characteristics of ketopiperazines, and it seemed that by the application of these tests the presence or absence of ketopiperazines in proteins could be detected. These tests were first applied to gelatin¹ and subsequently to casein.² Since the conditions of experiment first applied to gelatin were not conveniently applicable to casein and other proteins, the work on gelatin has been repeated under conditions identical with those applied to the other proteins.

¹ Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **74**, 715 (1927).

² Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **78**, 145 (1928).

³ Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, **63**, 661, (1925); *J. Gen. Physiol.*, **8**, 183 (1925); *J. Biol. Chem.*, **68**, 277 (1926); **70**, 219 (1926). Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **76**, 299 (1928).

⁴ Cf. Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **81**, 697 (1929).

In the present communication are given the results of the study of the action of alkali of different concentrations under uniform conditions on five proteins, namely albumin, casein, edestin, fibrin, and gelatin. In Tables I to V are given the changes in optical rotation under the influence of 0.2 *N*, 1.0 *N*, and 5.0 *N* sodium hydroxide. In Column 6 of Tables I to IV is given the degree of racemization calculated on complete racemization and in Column 7 the degree of racemization calculated on maximum observed racemization. Thus Column 6 gives the extent of racemization and Column 7 expresses in a way the speed of racemization.

Albumin, Casein, Edestin, Fibrin.

Action of 0.2 N Sodium Hydroxide.—From Tables I to IV it is seen definitely that these four proteins do not behave as would be expected of a mixture of ketopiperazines, inasmuch as under the influence of 0.2 *N* alkali not more than 15 per cent of racemization is obtained in 24 hours, whereas the minimum racemization at 20° observed on a ketopiperazine is 72 per cent and generally it is in the neighborhood of 85 per cent. These data on ketopiperazines were obtained from observations made on the anhydrides of dextro-alanyl-dextro-alanine, glycyl-levo-alanine, levo-prolyl-glycine, glycyl-dextro-valine, and glycyl-levo-leucine. After 4 days the racemization of the four proteins had proceeded to the extent of 17, 29, 27, and 22 per cent respectively. In the same interval of time and under the same conditions, the tetrapeptide glycyl-levo-alanyl-levo-alanyl-glycine showed a racemization of 19 per cent.

Thus the conduct of these proteins under the influence of 0.2 *N* alkali is more easily interpreted on the assumption of the peptide structure than on the basis of the ketopiperazine theory.

Action of 1.0 N Sodium Hydroxide.—Under the influence of alkali of this concentration there is observed in 24 hours racemization to the extent of 25, 41, 36, and 33 per cent respectively in these four proteins. In the same interval the degree of hydrolysis is 8, 4, 17, and 17 per cent respectively. The action of 1.0 *N* alkali has been tested on the anhydrides of dextro-alanyl-dextro-alanine, levo-alanyl-glycine, and levo-leucyl-glycine. Less than 7 per cent racemization was observed for levo-alanyl-glycine

anhydride and 20 per cent for levo-leucyl-glycine anhydride. The low rates of racemization of the anhydrides are due to the high rates of hydrolysis caused by 1.0 N alkali. In order that

TABLE I.
Racemization of Albumin.

	Time. (1)	Degree of hydrolysis by alkali. (2)	After acid hydrolysis.			Racemization calculated on complete racemization. (6)	Racemization calculated on maximum observed racemization. (7)
			Total N per cc. (3)	Amino N Total N. (4)	α_D corrected. (5)		
	<i>hrs.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>	<i>degrees</i>	<i>per cent</i>	<i>per cent</i>
Hydrolysis by 5.0 N HCl.	2			73.6	+2.12		
	4			77.6	+2.34		
	8			77.4	+2.31		
	24			77.4	+2.27		
	<i>days</i>						
Racemization by 0.2 N NaOH.	Control.		0.938	77.2	+2.33		
	1	3	0.858	74.8	+2.19	6	9
	2	5	0.763	76.4	+2.08	11	14
	4	6	0.763	74.2	+1.94	17	24
	7	8	0.885	73.8	+1.81	22	33
	15	12	0.830	75.4	+1.59	32	46
Racemization by 1.0 N NaOH.	Control.		0.942	76.2	+2.36		
	1	8	0.847	73.8	+1.78	25	36
	2	12	0.903	77.4	+1.65	30	44
	4	18	0.888	75.9	+1.24	47	70
	7	22	0.861	73.6	+0.95	60	88
	15	27	0.878	76.7	+0.77	67	99
Racemization by 5.0 N NaOH.	Control.		0.928	75.4	+2.45		
	1	20	0.886	74.9	+1.44	41	63
	2	32	0.645	77.1	+1.31	47	71
	4	39	0.861	76.7	+1.13	54	83
	7	46	0.702	75.5	+0.88	64	98
	15	49	0.868	74.2	+0.85	65	100

ketopiperazines may be racemized to 25 to 36 per cent in 24 hours, they need to possess much greater stability than those tested up to the present time. On the other hand, the tetrapeptide glycyl-

levo-alanyl-levo-alanyl-glycine, after 4 days standing with 0.5 N alkali, suffered racemization to the extent of 33 per cent, and, indeed, more stable peptides may show under the same conditions

TABLE II.
Racemization of Casein.

	Time.	Degree of hydrolysis by alkali.	After acid hydrolysis.			Racemization calculated on complete racemization.	Racemization calculated on maximum observed racemization.
			Total N per cc.	Amino N Total N.	α_D corrected.		
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
	hrs.	per cent	mg.	per cent	degrees	per cent	per cent
Hydrolysis by 5.0 N HCl.	2			69.8	+1.60		
	4			75.7	+1.86		
	8			76.0	+1.81		
	24			75.8	+1.76		
	days						
Racemization by 0.2 N NaOH.	Control.		0.897	75.9	+1.85		
	1	1	0.806	72.2	+1.68		10
	2	3	0.790	73.3	+1.55	9	18
	4	4	0.833	74.6	+1.31	29	32
	7	4	0.847	73.1	+1.23	34	37
	15	8	0.766	73.2	+1.01	45	50
Racemization by 1.0 N NaOH.	Control.		0.913	76.1	+1.87		
	1	4	0.878	75.9	+1.10	41	46
	2	7	0.665	74.0	+0.88	53	59
	4	9	0.831	75.3	+0.51	73	81
	7	11	0.860	74.3	+0.40	79	87
	15	21	0.801	74.5	+0.23	88	97
Racemization by 5.0 N NaOH.	Control.		0.890	75.6	+1.94		
	1	13	0.826	75.1	+0.77	60	69
	2	21	0.841	73.8	+0.60	69	79
	4	27	0.793	74.6	+0.41	79	91
	7	37	0.812	75.1	+0.34	83	95
	15	53	0.834	74.6	+0.25	87	100

even a higher degree of racemization. More recently Abderhalden⁵ and his coworkers have entered the field of work on the

⁵ Abderhalden, E., and coworkers, many papers in *Fermentforschung* and *Z. physiol. Chem.* since 1927.

action of alkali on peptides and they report complete racemization of a poly-leucyl-peptide on treatment with 1.0 N sodium hydroxide.

TABLE III.
Racemization of Edestin.

	Time. (1)	Degree of hydrolysis by alkali. (2)	After acid hydrolysis.			Racemization calculated on complete racemization. (6)	Racemization calculated on maximum observed racemization. (7)
			Total N per cc. ()	Amino N Total N (4)	α_D corrected. (5)		
	hrs.	per cent	mg.	per cent	degrees	per cent	per cent
Hydrolysis by 5.0 N HCl.	2			66.8	+1.25		
	4			67.9	+1.25		
	8			67.9	+1.26		
	24			68.2	+1.25		
	days						
Racemization by 0.2 N NaOH.	Control.		0.987	67.4	+1.26		
	1	7	0.983	67.4	+1.08	14	16
	2	7	0.959	67.9	+0.99	21	25
	4	11	0.986	68.3	+0.92	27	31
	7	15	0.927	68.4	+0.77	39	45
	15	16	0.982	67.9	+0.59	53	61
Racemization by 1.0 N NaOH.	Control.		1.001	67.7	+1.28		
	1	17	0.887	68.2	+0.82	36	42
	2	20	1.008	70.3	+0.63	51	59
	4	30	0.944	69.4	+0.43	66	77
	7	35	0.977	68.8	+0.32	75	87
	15	42	0.910	71.3	+0.23	82	95
Racemization by 5.0 N NaOH.	Control.		1.008	67.6	+1.35		
	1	29	0.878	69.1	+0.71	47	58
	2	46	0.962	68.7	+0.48	64	79
	4	58	0.970	69.7	+0.42	69	85
	7	65	0.868	70.6	+0.32	76	94
	15	82	0.847	73.9	+0.25	82	100

Thus again the degree of racemization of the four proteins by 1.0 N sodium hydroxide is more readily interpreted on the assumption of the peptide structure than on the ketopiperazine theory of protein structure. The progress of hydrolysis also harmonizes

better with the former view inasmuch as most ketopiperazines on treatment with 1.0 N alkali hydrolyze completely to the dipeptides

TABLE IV.
Racemization of Fibrin.

	Time. (1)	Degree of hydrolysis by alkali. (2)	After acid hydrolysis.			Racemization calculated on complete racemization. (6)	Racemization calculated on maximum observed racemization. (7)
			Total N per cc. (3)	Amino N Total N. (4)	α corrected. degrees (5)		
	<i>hrs.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>	<i>degrees</i>	<i>per cent</i>	<i>per cent</i>
Hydrolysis by 5.0 N HCl.	2			77.7	+2.09		
	4			80.1	+2.19		
	8			80.5	+2.18		
	24			79.2	+2.21		
	<i>days</i>						
Racemization by 0.2 N NaOH.	Control.		0.938	80.7	+2.18		
	1	6	0.704	78.1	+1.85	15	18
	2	9	0.832	79.7	+1.83	16	19
	4	11	0.854	80.3	+1.70	22	26
	7	12	0.721	78.5	+1.59	27	32
	15	15	0.768	78.5	+1.41	35	41
Racemization by 1.0 N NaOH.	Control.		0.952	79.6	+2.22		
	1	17	0.933	79.2	+1.49	33	39
	2	19	0.913	80.2	+1.28	42	50
	4	25	0.924	78.8	+1.01	55	65
	7	34	0.948	78.5	+0.77	65	78
	15	37	0.740	80.6	+0.58	74	88
Racemization by 5.0 N NaOH.	Control.		0.961	79.8	+2.35		
	1	35	0.858	78.4	+1.28	46	57
	2	44	0.867	80.3	+1.09	54	67
	4	51	0.885	78.3	+0.86	63	80
	7	56	0.854	80.7	+0.65	72	91
	15	67	0.858	81.0	+0.48	80	100

within 24 hours. It must be added, however, that in this respect also more data on synthetic compounds are much needed.

Action of 5.0 N Sodium Hydroxide.—The effect of alkali of this concentration is nearly the same as that described in the section

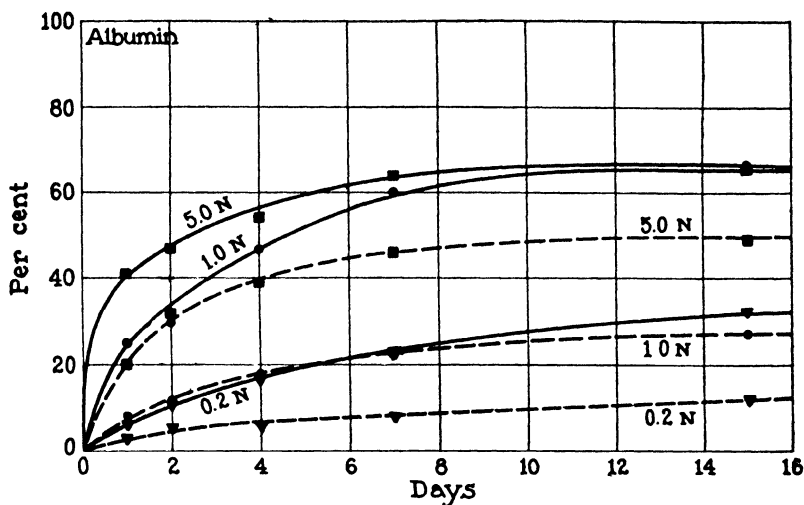


FIG. 1. Action of alkali on albumin. The degree of racemization calculated on complete racemization (solid lines) and the degree of hydrolysis (broken lines) are plotted against the time.

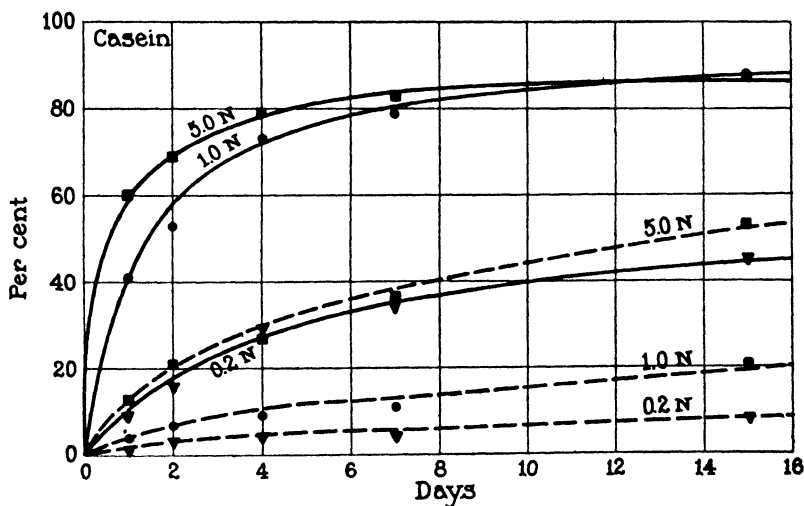


FIG. 2. Action of alkali on casein. The degree of racemization calculated on complete racemization (solid lines) and the degree of hydrolysis (broken lines) are plotted against the time.

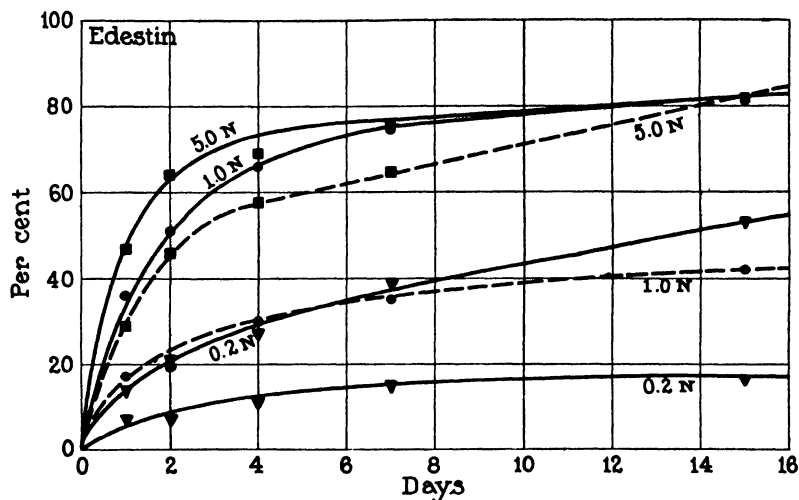


FIG. 3. Action of alkali on edestin. The degree of racemization calculated on complete racemization (solid lines) and the degree of hydrolysis (broken lines) are plotted against the time.

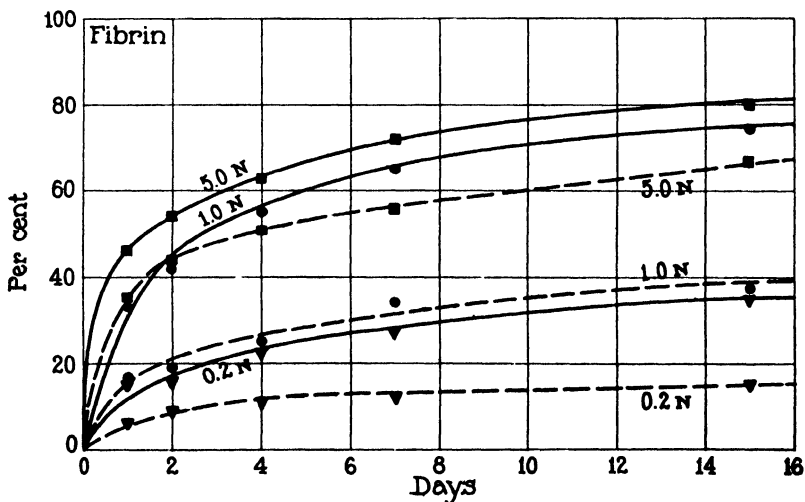


FIG. 4. Action of alkali on fibrin. The degree of racemization calculated on complete racemization (solid lines) and the degree of hydrolysis (broken lines) are plotted against the time.

above. The course of racemization is only slightly higher and the degree of hydrolysis is considerably higher than under the influence

TABLE V.
Racemization of Gelatin.

	Time.	Degree of hydrolysis by alkali.	After acid hydrolysis.		
			Total N per cc.	Amino N Total N	α_D^{25} corrected.
	(1)	(2)	(3)	(4)	(5)
	<i>hrs.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>	<i>degrees</i>
Hydrolysis by 5.0 N HCl.	2			67.9	-0.53
	4			68.2	-0.35
	8			68.5	-0.31
	24			68.3	-0.27
	<i>days</i>				
Racemization by 0.2 N NaOH.	Control.		1.124	68.4	-0.36
	1	3	1.105	68.0	-0.86
	2	6	1.101	69.2	-1.01
	4	10	1.110	68.6	-0.98
	7	14	1.098	68.3	-1.02
	15	26	1.099	69.5	-1.13
Racemization by 1.0 N NaOH.	Control.		1.088	69.3	-0.32
	1	30	1.112	68.5	-0.87
	2	43	1.110	69.3	-0.80
	4	56	1.042	69.0	-0.71
	7	65	0.942	67.8	-0.65
	15	80	1.075	70.3	-0.67
Racemization by 5.0 N NaOH.	Control.		1.094	69.1	-0.23
	1	79	1.029	68.1	-0.08
	2	85	1.003	69.2	-0.01
	4	89	1.055	68.9	+0.01
	7	94	1.000	69.0	0
	15	101	1.040	70.4	-0.036*

* Measured on a polarimeter reading to 0.001°.

of 1.0 N alkali, yet in no case was the rate of hydrolysis as high as in the case of ketopiperazines.

Gelatin.

From Table V it is seen that gelatin occupies a separate position from that of the other proteins. In this protein the levorotatory amino acids predominate and, furthermore, under the influence of 0.2 *N* alkali the racemization of the dextrorotatory amino acids proceeds at higher rate, so that the levorotation of the total amino acids increases. With 1.0 *N* and 5.0 *N* alkali the levorotatory amino acids also undergo racemization. It is therefore difficult to express the progress of racemization in percentages of

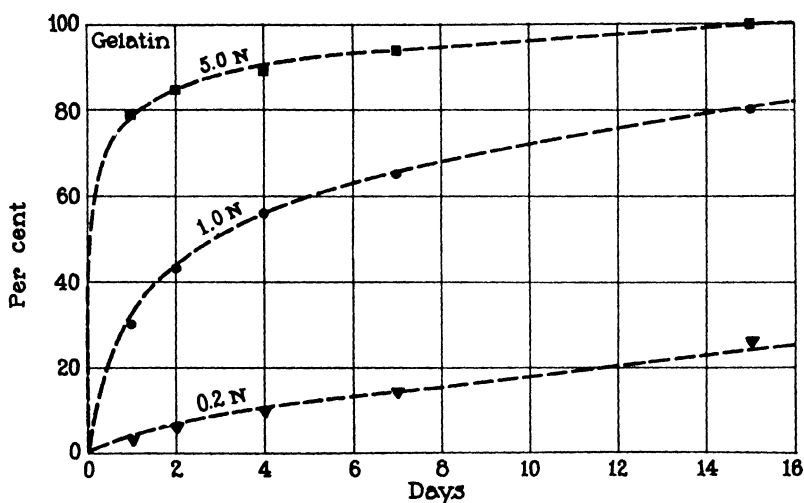


FIG. 5. Action of alkali on gelatin. The degree of hydrolysis (broken lines) is plotted against the time.

total racemization or in percentages of maximum observed racemization.

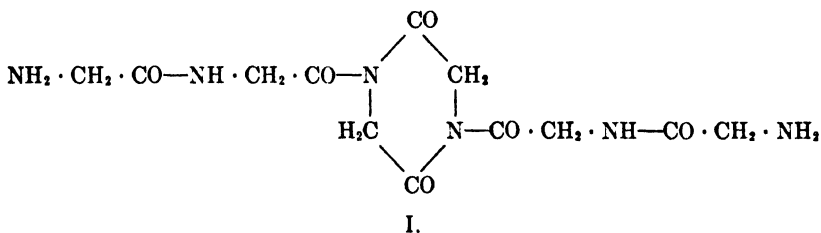
The unique behavior of gelatin is characterized by still another fact; namely, that with every concentration of alkali practically the maximum racemization obtainable by that concentration of alkali is attained in 24 hours. In a way this behavior may be interpreted as indicating the presence of ketopiperazines. On the other hand the fact that racemization progresses to a higher degree with 1.0 *N* and 5.0 *N* alkali again seems to indicate the presence also of polypeptide structures even in gelatin.

*Conclusions Regarding the Structure of Proteins Based on the
Progress of Racemization.*

Thus the progress of racemization and of hydrolysis of the four proteins albumin, casein, edestin, and fibrin under the influence of alkali of varying concentration offers no evidence in favor of the theory of the ketopiperazine structure of the protein molecule.

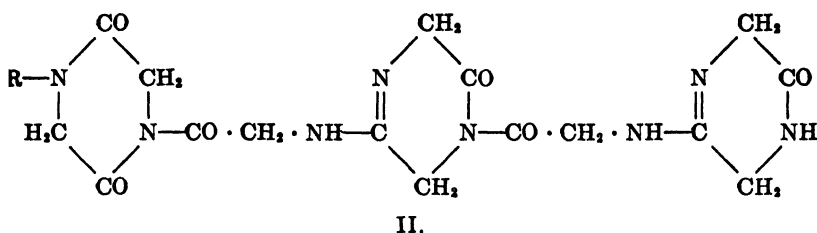
The behavior of gelatin is somewhat different from that of the other proteins. *It is, however, certain that the molecule of gelatin is not composed of ketopiperazines only.*

The assumption of the presence of ketopiperazines and peptides in the protein molecule raises the question as to the possible number of ketopiperazine rings in a chain of amino acids. It is evident that the keto (lactam) form of the ketopiperazines does not permit the presence of more than one ring in a chain of any length, provided that only the α -amino group and only the carboxyl group neighboring to the amino group take part in the chain formation. Formula I brings out the reasons for this statement. In order to permit a high



proportion of ketopiperazines in the proteins, it would be necessary to assume that proteins are composed of small chains of the type given in Formula I, held together by molecular forces. Molecular structures composed in this manner should exhibit a higher $\frac{\text{amino nitrogen}}{\text{total nitrogen}}$ ratio than is generally observed in proteins.

Chains of amino acids containing more than one ketopiperazine ring are possible only if the greater part of the ketopiperazines are formulated with a lactim structure (Formula II). Such chains would require a $\frac{\text{nitrogen}}{\text{oxygen}}$ ratio much higher than that observed in proteins.



* Thus it seems that even in the case in which the progress of racemization does not exclude the possibility of the theory of the mixed structure of proteins, theoretical considerations warn against immediate acceptance of the theory at this date. Final decision, however, should be deferred until more data have been obtained on synthetic ketopiperazines and peptides.

Other Conclusions from the Progress of Racemization and Hydrolysis of Proteins.

The present day information on the differences in the structure of proteins is limited to a comparison of the number, of the character, and of the ratios of the component amino acids. Comparatively little is known about the arrangement of the amino acids in the peptide chains (if the peptide structure is accepted, or of their arrangement in anhydrides if the anhydride theory should gain ground). The only attempt to obtain such information is contained in the investigations of Dakin and his coworkers (see Table VI). Dakin's investigations demonstrate the fact that differences in the arrangement of amino acids actually exist. However, these investigations were not extended sufficiently to detect the differences. This task still remains a very difficult one. The path chosen by us consists of the study of the rates of racemization and hydrolysis by alkali, on one hand, of proteins and the so called proteoses and peptones, and on the other hand, of peptides and ketopiperazines prepared synthetically and therefore of known structure.

The differences in the rates of hydrolysis by alkali of some dipeptides are shown in Table VII. From this table it is evident that the rate of alkaline hydrolysis differs with the structure of the peptide. More extensive data of a purely qualitative character have been accumulated in the last 2 years by Abderhalden⁵ and

TABLE VI.
Racemization of Proteins According to Dakin and His Coworkers.

Amino acid.	Gelatin.*	Casein.†	Caseinogen‡ (cow).	Caseinogen‡ (sheep).	Albumin§ (hen).	Albumin§ (duck).
Alanine.....	Partial.	Partial (?)	Partial (?)	Partial (?)	None.	None.
Valine.....	Complete.	"	Partial.	Partial.	Partial.	Partial.
Leucine.....			"	"	Mostly race- mized.	Mostly active.
Phenylalanine.....	"	Complete.	Complete.	Complete.	Complete.	Complete.
Tyrosine.....	(?)	"	"	None.	"	"
Serine.....	(?)			None.	Mostly race- mized.	Mostly race- mized.
Proline.....	None.	None.	None.	None.	Mostly race- mized, some active.	Complete.
Aspartic acid.....	Complete.	Complete.	Complete.	Complete.	Complete.	Complete.
Glutamic acid.....	None.	"	"	Partial.	Complete.	"
Arginine.....	Complete.	"	"	Complete.	None.	None.
Histidine.....	"	"	"	"	Complete.	Mostly active.
Lysine.....	None.	"	"	None.	"	Complete.

* Dakin, H. D., *J. Biol. Chem.*, **13**, 357 (1912-13).

† Dakin, H. D., and Dudley, H. W., *J. Biol. Chem.*, **15**, 263 (1913).

‡ Dudley, H. W., and Woodman, H. E., *Biochem. J.*, **9**, 97 (1915).

§ Dakin, H. D., and Dale, H. H., *Biochem. J.*, **13**, 248 (1919).

TABLE VII.
*Rates of Hydrolysis of Dipeptides by Alkali at 25° **

	<i>k</i> · 10 ³
Glycyl-glycine.....	152
Glycyl-alanine.....	37
Alanyl-glycine.....	32
Alanyl-alanine.....	7.6
Glycyl- α -aminoisobutyric acid.....	0
Glycyl-phenylaminoacetic acid.....	22
Glycyl-phenylalanine.....	7.9
Glycyl-phenylmethylaminoacetic acid.....	0

* Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **82**, 167 (1929).

TABLE VIII.
Amino Acid Content of Protein.

Amino acid.	Albu- min.*	Casein.†	Ede- tin.‡	Fibrin.§	Gelatin.
Glycine.....	0	0.45	3.80	3.0	25.5
Alanine.....	8.1	1.85	3.60	3.6	8.7
Valine.....	2.5	7.93	6.20	1.0	
Leucine.....	10.7	7.92	20.9	15.0	7.1
Isoleucine.....		1.43			
Phenylalanine.....	5.17	3.88	3.09	2.5	1.4
Tyrosine.....	1.77	5.70	2.13	3.5	0.01
Serine.....		0.43	0.33	0.8	0.4
Proline.....	3.56	8.70	4.10	3.6	9.5
Hydroxyproline.....		0.23	2.0		14.1
Aspartic acid.....	2.2	4.10	4.50	2.0	3.4
Glutamic acid.....	9.1	21.77	18.74	10.4	5.8
Hydroxyglutamic acid.....		10.50			
Cystine.....	0.3	0.02	1.0	1.17	
Tryptophane.....	+	1.70	0.38		
Arginine.....	4.91	4.84	14.17	4.7	8.2
Histidine.....	1.71	3.39	2.19	6.4	0.9
Lysine.....	3.76	7.72	1.65	11.1	5.9
	53.78	92.56	88.78	68.77	90.91

* Strauss, E., and Collier, W. A., in Oppenheimer, C., and Pincussen, L., *Tabulæ Biologicæ*, Berlin, **3**, 258 (1926).

† Gortner, R. A., in Sutermeister, E., *Casein and its industrial applications*, New York, 19, (1927).

‡ See (*) p. 265.

§ See (*) p. 258.

|| Dakin, H. D., *J. Biol. Chem.*, **44**, 499 (1920).

his coworkers. Sufficient data on the differences in the rates of racemization of peptides as a function of structure are still lacking, but the observations recorded in Tables I to V and in Figs. 1 to 5 can already be interpreted on the assumption that the differences in the rates of hydrolysis and of racemization are at least in a great part due to differences in the proportions of the amino acids contained in them. Thus by a comparison of Tables VII and VIII, gelatin would be expected to exhibit the lowest resistance towards the hydrolytic action of alkali.

On the other hand, the conclusion that not only the character of the amino acids but also their arrangement determines the stability of proteins can be drawn from the behavior of casein and edestin. In both, the proportion of glutamic acid is high. In edestin the proportion of leucine is higher than in casein. According to Abderhalden the stability of leucyl-glutamic acid is greater than that of glycyl-glutamic acid. Edestin, however, is hydrolyzed at a much higher rate than casein. The probability then is that in edestin the proportion of leucyl-glutamic acid is lower than in casein. This instance is pointed out only to indicate the process of reasoning through which information will eventually be obtained on the arrangement of amino acids in the protein molecules.

EXPERIMENTAL.

General Procedure.

Acid Hydrolysis.—Samples of 1.0 gm. (corrected for moisture) of protein were sealed in Pyrex test-tubes with 10.0 cc. of 5.0 N hydrochloric acid and the quantity of norit shown by preliminary experiments to be sufficient to absorb the colored matter formed during hydrolysis. The tubes were then suspended for definite periods of time in copper gauze containers in a glycerol thermostat at 125° ($\pm 1.0^\circ$).

The test-tubes were cooled and opened. The contents were filtered by suction, and the charcoal was washed with distilled water. The filtrate and washings were diluted to 25.0 cc. in volumetric flasks. These solutions were often slightly colored. The rotations were read at 25° for the wave-length 5892 Å. in 4 dm. tubes.

Samples of 4.0 cc. of the rotation solutions were neutralized to

phenolphthalein and diluted to 25.0 cc. Total nitrogen (Kjeldahl) was determined on 10.0 cc. portions and amino nitrogen (micro Van Slyke, 15 minutes shaking) on 2.0 cc. These dilutions were so chosen as to give fairly large readings in the analytical procedure.

The rotations were then corrected to the theoretical value of total nitrogen, calculated from the per cent of nitrogen in the original sample and the dilution.

Racemization.—Samples of 1.0 gm. of protein, 1 drop of octyl alcohol, and 25.0 cc. of standard alkali (0.2 N, 1.0 N, and 5.0 N) were allowed to stand for definite periods of time at 25° in tightly stoppered Erlenmeyer flasks.

The solutions were neutralized with hydrochloric acid. A flocculent precipitate of "racemized" protein was thrown down on neutralization (except in the case of gelatin). These solutions (including the precipitates) were then concentrated to dryness under reduced pressure. For this purpose Pyrex test-tubes were used, equipped with a wide distilling head carrying a dropping funnel and a wide capillary (capped with a rubber policeman to diminish the air admitted) reaching to the bottom of the tube. The reduced pressure distillations were carried out on the water pump at 50-60°. The solutions were introduced gradually through the dropping funnel. Octyl alcohol was used sparingly and ethyl alcohol more liberally to prevent frothing over. In most cases some loss occurred; also, some protein collected in the distilling head. When the protein solutions had been evaporated to dryness, the residues were completely hydrolyzed by the following procedure.

The quantity of norit sufficient for decolorization was added to each test-tube. 10 cc. of 5.0 N hydrochloric acid were then added; part of the acid was used to rinse the distilling head and the remainder was used to wash down the sides of the tube. The test-tubes were then sealed and hydrolyzed 4 hours at 125°. This time has been found most suitable for all the proteins thus far studied; *i.e.*, it gives a maximum amino nitrogen ratio and a constant rotation. After hydrolysis was complete, the rotations and amino nitrogen ratios were determined exactly as described under "Acid Hydrolysis."

Control experiments were run as follows: Samples of protein were hydrolyzed with 10.0 cc. of 5.0 N hydrochloric acid and the

quantities of sodium chloride equivalent to the alkali used in the racemization experiments. The procedure was the same as that employed for acid hydrolysis.

Alkaline Hydrolysis.—Samples of 2.0 gm. of protein were dissolved in 50.0 cc. of standard alkali (0.2 N, 1.0 N, and 5.0 N); 2 drops of octyl alcohol were added to prevent foaming. These solutions were allowed to stand at 25°. At definite time intervals samples of 4.0 cc. were removed, neutralized to phenolphthalein, and diluted to 25.0 cc. Total nitrogen (Kjeldahl) was determined on 10.0 cc. samples and amino nitrogen (micro Van Slyke, 30 minutes shaking) on 2.0 cc. samples.

For the calculation of the degree of hydrolysis (H), the following formula was used, in which r_0 is the amino ratio of the original sample of protein, r_t the amino ratio at the given time interval, and r_∞ the amino ratio obtained by complete hydrolysis with acid.

$$H = \frac{r_t - r_0}{r_\infty - r_0}$$

Albumin.

The ovalbumin was recrystallized three times, coagulated at the isoelectric point, washed thoroughly, and dried in a vacuum desiccator at room temperature. The final product gave the following analysis (calculated as dry substance).

C 51.01, H 6.91, S 2.54, P 0, N 15.51.

Ash 0. Moisture 14.07.

A second, smaller sample, prepared independently for comparison, was dried under reduced pressure at 50°.

C 51.11, H 6.57, S 2.58, P 0, N 15.44.

Ash 0. Moisture 5.34.

It seems probable, from a comparison of these analyses with analyses recorded in the literature, that our material still contained ammonium sulfate as an impurity. For the present investigation, however, this fact is of no importance.

The data on the racemization of albumin are given in Table I

Acid Hydrolysis.—For decolorization 0.4 gm. of norit was used with each sample. The rotations were corrected to values corre-

sponding to 0.993 mg. of nitrogen per cc. in the analyzed solutions.

Racemization.—The racemization was studied by the procedure given above.

Alkaline Hydrolysis.—In calculating the degree of hydrolysis, r_0 was taken as 6.0 per cent and r_∞ as 77.4 per cent.

Casein.

The casein is the same sample as that used previously.² It gave the following analysis (calculated as dry substance).

C 53.56, H 6.91, P 0.63, S 1.13, N 14.94, NH_2 0.80.
Ash 2.16. Moisture 8.41.

The data on the racemization of casein are given in Table II.

Acid Hydrolysis.—The values reported in the previous paper have been checked. For decolorization 0.4 gm. of norit was used with each sample. The rotations were corrected to values corresponding to 0.956 mg. of nitrogen per cc. in the analyzed solutions.

Racemization.—The results are essentially the same as those reported previously, except that 0.2 N alkali has been used instead of 0.5 N.

Alkaline Hydrolysis.—The results are essentially the same as those reported previously, except that 0.2 N alkali has been used instead of 0.5 N. In calculating the degree of hydrolysis, r_0 was taken as 5.4 per cent and r_∞ as 75.8 per cent.

Edestin.

The edestin, recrystallized several times, gave the following analysis (calculated as dry substance).

C 50.91, H 6.89, S 1.01, N 18.43, NH_2 0.33.
Ash 0.61. Moisture 6.58.

The data on the racemization of edestin are given in Table III.

Acid Hydrolysis.—Samples of 0.5 gm. were hydrolyzed with 10.0 cc. of 5.0 N hydrochloric acid; 0.4 gm. of norit was used for decolorization. For analysis 7.5 cc. samples of the rotation solutions were neutralized and diluted to 25.0 cc. The rotations were corrected to values corresponding to 1.106 mg. of nitrogen per cc. in the analyzed solutions.

Racemization.—Samples of 0.5 gm. were dissolved in 12.5 cc. of standard sodium hydroxide.

Alkaline Hydrolysis.—In calculating the degree of hydrolysis, r_0 was taken as 1.8 per cent and r_∞ as 67.9 per cent.

Fibrin.

The fibrin, prepared from beef blood, gave the following analysis (calculated as dry substance).

C 51.67, H 6.51, S 1.07, N 17.73, NH_2 0.28.

Ash 1.11. Moisture 6.50.

The data on the racemization of fibrin are given in Table IV.

Acid Hydrolysis.—For decolorization 1.0 gm. of norit was used with each sample. The rotations were corrected to values corresponding to 1.135 mg. of nitrogen per cc. in the analyzed solutions.

Racemization.—The racemization was studied by the procedure given above.

Alkaline Hydrolysis.—In calculating the degree of hydrolysis, r_0 was taken as 1.6 per cent and r_∞ as 80.0 per cent.

Gelatin.

The gelatin, prepared by the same method as the sample used previously,^{1,6} gave the following analysis (calculated as dry substance).

C 50.86, H 6.32, N 18.03, NH_2 0.58.

Moisture 15.63.

The data on the racemization of gelatin are given in Table V.

Acid Hydrolysis.—For decolorization 0.3 gm. of norit was used with each sample. The rotations were corrected to values corresponding to 1.154 mg. of nitrogen per cc. in the analyzed solutions.

The time chosen for acid hydrolysis in the racemization experiments was 4 hours. While acid hydrolysis was apparently complete in most cases in 2 hours, this period was found to be a dangerous minimum, since in several experiments comparatively large deviations in both rotations and amino nitrogen ratios were observed.

⁶ We are indebted to Dr. J. H. Northrop for the sample of purified gelatin used in these experiments.

It should be mentioned that there is apparently an appreciable racemization at this temperature of some of the amino acids (probably proline and hydroxyproline) on more prolonged heating.

Racemization.—The racemization was studied by the procedure given above.

Alkaline Hydrolysis.—In calculating the degree of hydrolysis, r_0 was taken as 3.2 per cent and r_∞ as 68.3 per cent.

Stability of Optically Active Amino Acids towards Alkali.

In a preceding paper⁷ of this series it was found that when dextro-alanine was allowed to stand 35 days at 25° with 0.1 N, 0.2 N, or 1.0 N sodium hydroxide, no change occurred in the

TABLE IX.
Rotations of Amino Acids after Standing in Alkaline Solution

Amino acid.	1.0 N NaOH		$[\alpha]_D^{25}$ 5.0 N HCl.
	α_D^{25} original	α_D^{25} after 15 days	
	degrees	degrees	degrees
Dextro-alanine .	+0 20	+0.19	+14 7
Levo-valine .	-0 60	-0 61	-27 0
Levo-leucine .	+0 29	+0 29	+17 7
Dextro-isoleucine .	+0 36	+0 34	+19 2
Levo-tyrosine .	-0 55	-0 52	- 8 0
Levo-aspartic acid .	-0 13	-0.12	+23 5
Dextro-glutamic acid .	+0.42	+0 41	+30 5
Levo-histidine .	-0 44	-0 44	-11 7
Levo-hydroxyproline .	-2 56	-2 59	-46 5

rotation. This observation has now been extended to a large list of optically active amino acids. These acids were so chosen as to include representatives of the different types of amino acids found in proteins.

Samples of 0.200 gm. of each acid were made up to 10.0 cc. with 1.0 N NaOH; the rotations were read at 25° in 2.00 dm. tubes for the wave-length $\lambda = 5892 \text{ \AA}$. The solutions were sealed in Pyrex test-tubes and were allowed to stand 15 days at 25°; the rotations were then read. The data are recorded in Table IX. The specific rotations of the samples in 2 per cent solution in 5.0 N hydrochloric acid are also given.

⁷ Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, **70**, 219 (1926).

A NEW CASE OF WALDEN INVERSION IN THE HEXOSE SERIES.

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(Received for publication, February 9, 1929.)

The occurrence of Walden inversion in the hexoses was first observed by Fischer and Tiemann¹ when they prepared chitonic and chitaric acids from chitose and chitosaminic acid respectively. The surmise of Fischer was confirmed by Levene and La Forge² when they established the configurations of these two acids. The Walden inversion in this reaction accompanies ring formation between carbon atoms 2 and 5. Levene and La Forge³ have later shown that Walden inversion with a ring formation between carbon atoms 2 and 5 is a frequent occurrence in the 2-amino-hexose derivatives.

Isomeric to the 2,5-anhydrohexoses are the 3,6-anhydro forms, the first substance of this nature being obtained by Fischer and Zach.⁴ However, it remained uncertain whether or not the anhydro sugar retained the configuration of glucose. Levene and Sobotka⁵ later prepared a 3,6-anhydro sugar from 3-amino-hexose. The osazones from these two sugars were found to be isomeric, the melting point of the former being higher by 20° than that of the latter. On the basis of this difference in the melting points, Levene and Sobotka surmised that the higher melting osazone has the configuration of glucosazone and the lower melting, that of allosazone. Very recently Ohle, von Vargha, and

¹ Fischer, E., and Tiemann, F., *Ber. chem. Ges.*, **27**, 139 (1894).

² Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **21**, 351 (1915).

³ See monograph Levene, P. A., *Hexosamines and mucoproteins*, London and New York, 1925.

⁴ Fischer, E., and Zach, K., *Ber. chem. Ges.*, **45**, 456 (1912).

⁵ Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **71**, 181 (1926-27).

Erlbach⁶ obtained the anhydrohexose of Fischer and Zach through the process of saponification of di-*p*-toluenesulfomonoacetone glucose, and have corroborated in this manner the assumption of a 3,6-anhydro structure for the substance of Fischer and Zach.

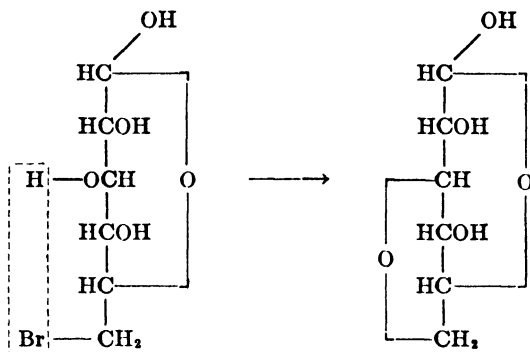
Recently we obtained from fructose-3-phosphoric acid the osazone isomeric to the one obtained from the Fischer and Zach anhydro sugar. In this case the hydrolysis of the phosphoric acid occurred simultaneously with the osazone formation. The osazone was similar to that prepared by Levene and Sobotka from 3-aminohexose; it was very soluble in cold methyl or ethyl alcohol, melted at 165-168°, crystallized in long curved needles, and was levorotatory.

In order to compare the properties of this osazone with that of Fischer and Zach, the latter substance was prepared according to the directions of these authors with the one exception that the 6-bromoglucose was derived from levo-glucosan according to the method of Karrer and Smirnoff.⁷ The osazone prepared from the Fischer and Zach 3,6-anhydro sugar was very sparingly soluble in cold methyl alcohol, melted at 188-190°, crystallized in short lemon-yellow prisms, and was levorotatory. In the parent substances of each of these osazones the carbon atoms 3, 4, and 5 had configurations identical with those in glucose and in both cases the ring formation involved a reaction between carbon atoms 3 and 6. Inasmuch as carbon atom 6 is symmetric, the isomerism of the two osazones can be explained only on the assumption of differences in the configurations of carbon atom 3. From this it follows that one substance is the osazone of the 3,6-anhydroglucose and the other is that of 3,6-anhydroallose. On comparing the properties of the osazones as given above it is easily seen that the differences between them are very analogous to those observed in the pair glucosazone and allosazone. It is therefore warranted to regard the osazone obtained from the fructose-3-phosphoric acid as 3,6-anhydroallosazone. From this conclusion it follows that the hydrolysis of the phosphoric group in fructose-3-phosphoric acid during osazone formation is accompanied by Walden inversion. The observation that 6-bromoglucose and

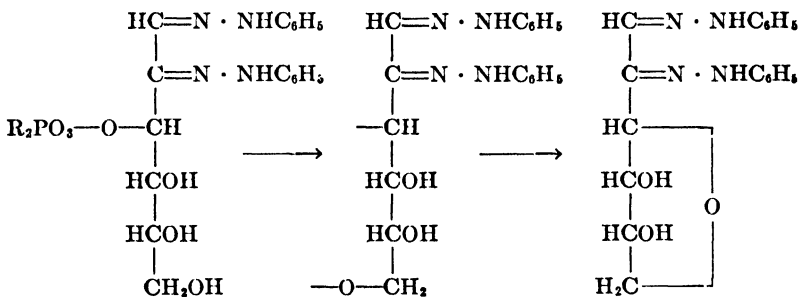
⁶ Ohle, H., von Vargha, L., and Erlbach, H., *Ber. chem. Ges.*, **61** B, 1211 (1928).

⁷ Karrer, P., and Smirnoff, A. P., *Helv. Chim. Acta*, **5**, 124 (1922).

6-toluenesulfoglucose lead to ring formation without Walden inversion, whereas fructose-3-phosphoric acid forms a 3,6-anhydro derivative accompanied by Walden inversion, emphasizes the fact that the inversion occurs only in reactions of substitution on the asymmetric carbon atom. The two respective reactions of ring formation may then be represented by the following figures.



I.



II.

It might be assumed that the inversion occurred in the process of ester formation. This assumption, however, is not probable for the reason that the process of ring formation in the case of the substance of Fischer and Zach is not associated with a Walden inversion and the reaction of ring formation is very analogous to that of substitution on carbon atom 3.

In connection with the conduct of the fructose-3-phosphoric acid, attention may be called to the suggestion advanced by

Robinson⁸ in the summer of 1927, according to which the various isomeric hexoses discovered in nature are formed through the intermediary phase of the formation of phosphoric esters which on hydrolysis suffer Walden inversion.

EXPERIMENTAL.

3,6-Anhydroglucose.—Anhydroglucose was prepared from triacetyl-1,6-dibromoglucose according to the procedures of Fischer and Zach.⁴ The acetodibromoglucose was in turn prepared from triacetyl-levo-glucosan by Karrer and Smirnoff's⁷ procedure. The details of this preparation were as follows:

To 15 gm. of well dried, finely pulverized aceto-levo-glucosan (m.p. 110°, light yellow in color) were added 50 gm. of finely ground phosphorus pentabromide in a flask attached with a ground glass joint to a reflux condenser. The flask was immersed in a water bath and shaken constantly. The bath was gradually warmed to 95° and at about 65° the mixture acquired an orange color—probably due to the liberation of bromine. Hydrogen bromide and a little bromine soon began to escape through the condenser and at the same time the mixture tended to solidify. Above 90° the mixture became quite viscous. The temperature was maintained at 90–95° for 5 minutes with continued shaking. On cooling, the reaction mixture became semisolid. It was transferred to 300 cc. of ice water and well triturated. The water was sucked off and the washing was thrice repeated. After filtering with suction, the product was thoroughly dried over phosphorus pentoxide in a vacuum desiccator. The dried material was twice extracted with a little ether and redried. Yield 17 gm. With silver carbonate and methyl alcohol it yielded a nicely crystalline aceto-6-bromomethylglucoside.

3,6-Anhydroglucose Osazone.—The osazone was prepared from the anhydroglucose by the procedure of Fischer and Zach.⁴ It was crystallized from hot methyl alcohol and analyzed as follows:

4.125 mg. substance: 9.600 mg. CO₂ and 2.170 mg. H₂O.

4.530 " " : 0.644 cc. N₂ (758 mm. and 22°).

C₁₃H₂₀O₅N₄. Calculated. C 63.47, H 5.92, N 16.47.

Found. " 63.46, " 5.88, " 16.40.

$$[\alpha]_D^{25} = \frac{-0.85^\circ \times 100}{0.5 \times 1.00} = -170^\circ.$$

⁸ Robinson, R., *Nature*, **120**, 656 (1927).

The pure product was only slightly soluble in cold methyl alcohol. It melted at 188–190° in a sealed tube and did not effervesce on further heating.

Fructose-3-Phosphoric Acid.—The preparation of the phosphate will be described in detail in a later communication; at present it is only necessary to state that α -diacetone fructose was phosphorylated in anhydrous pyridine with phosphorus oxychloride and isolated as the barium salt. This salt was subjected to mild acid hydrolysis which removed the acetone groups, giving a good yield of a pure white barium salt which analyzed well.

Osazone from Fructose-3-Phosphoric Acid.—The barium salt of the 3-fructose phosphoric acid was dissolved in water, cooled in ice, and the barium quantitatively removed with sulfuric acid. After centrifuging, a portion equivalent to 6 gm. (0.015 mols) of dry barium salt was diluted to a volume of 100 cc. and 4 cc. of glacial acetic acid and 6 cc. of phenylhydrazine (0.06 mols) were added. The mixture was heated in a boiling water bath. Every few minutes it was removed and cooled and the osazone filtered with suction. The heating was continued until practically no more osazone was formed (about 1 hour). The combined product was dried in a vacuum desiccator. Yield 2.8 gm. (55 per cent of the theoretical).

The dry material was twice recrystallized from aqueous pyridine and then once from methyl alcohol. The analysis was nearly perfect for an anhydrohexose osazone.

4.940 mg. substance: 11.495 mg. CO₂ and 2.590 mg. H₂O.

5.351 " " : 0.759 cc. N₂ (762 mm. and 22°).

C₁₈H₂₀O₃N₄. Calculated. C 63.47, H 5.92, N 16.47.

Found. " 63.45, " 5.86, " 16.44, P None.

$$[\alpha]_D^{20} = \frac{-0.55^\circ \times 100}{0.5 \times 0.8} = -138^\circ.$$

The pure product was quite soluble in cold methyl alcohol. In a sealed tube it softened at 160° and melted at 165–168°.

THE ACTIVITY COEFFICIENTS OF CERTAIN ACID-BASE INDICATORS.*

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(Received for publication, December 29, 1928.)

At an early stage in the development of the colorimetric method, Küster (1), Van Cleeff (2), Acree (3), von Szyszkowski (4), and Michaelis and Rona (5) recognized and pointed out the fact that the salt content of the solution influenced the reaction of the indicator. The color of the same indicator in the same buffer solution could be changed by addition of neutral salts. Sørensen and Palitzsch (6) called attention to the fact that it was necessary to consider separately, the "neutral salt effect" on the buffer itself, and on the indicator color. In 1914, Bjerrum (7) suggested that this double effect had to do with the change in the dissociation constant of the buffer, and also of the indicator. He found that the change in the logarithm of the indicator dissociation constant was proportional to the variation in salt concentration.

The literature covering the work done on "salt errors" of indicators will be found in the texts and monographs of Thiel (8), Bjerrum (7), Prideaux (9), Michaelis (10), Kolthoff (11), and Clark (12), and will be referred to in connection with the development of the text of this paper. Kolthoff, in 1926, summarized the results in saying that, "Various theories have been advanced to explain the salt error, but none of them is adequate for the quantitative interpretation of the behavior of every indicator." The indicator error caused by different salts has been found to vary in an apparently irrational manner. The question has therefore been allowed to rest as a purely empirical matter. "Salt error" has been correlated with percentage salt, equivalent concentration, molecular concentration, and sodium ion concentration. The effect of salt has been determined in most cases at such

* A preliminary report of this work appeared under the above title in the Proceedings of the American Society of Biological Chemists, *J. Biol. Chem.*, **78**, p. lxvii (1928).

high concentrations of electrolyte as to be of little help in biological work, where solutions are seldom used of an electrolyte strength exceeding that of 0.9 per cent NaCl.

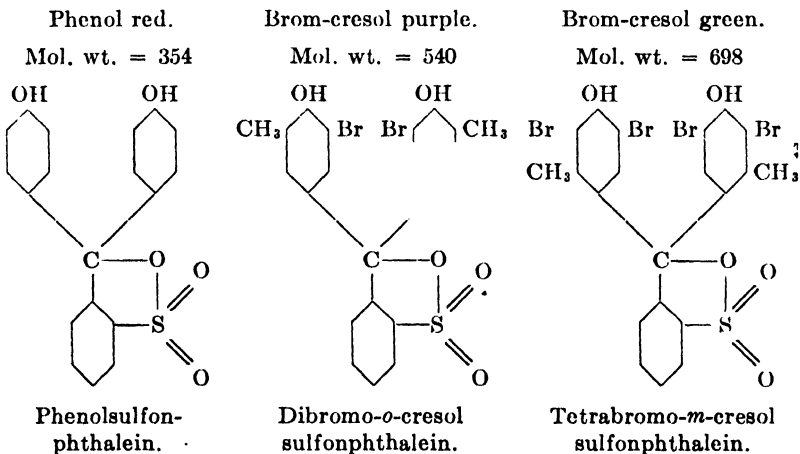
It is important to note, however, that in 1921, Brönsted (13), on the basis of some of Sørensen's old data, suggested that the behavior of some indicators, including phenolphthalein and neutral red, might be due to the effect of interionic forces in electrolytic solutions. He obtained good agreement between Sørensen's data and the theoretical equations which he and Bjerrum had developed on the basis of the former's theory of complete dissociation.

In the present paper we have attempted to utilize recent advances in the theory of solutions to study indicator behavior in the presence of salts. The indicators studied have been brom-cresol green, brom-cresol purple, and phenol red, covering the pH range from 4.0 to 8.2. A theory, originally suggested by Brönsted, has been further developed and applied to the systematic quantitative investigation of the change in color of these indicators under the influence of certain salts.

THEORETICAL.

The theoretical aspects of indicator color change are sufficiently discussed in the texts referred to above, so that it will be necessary to present here only a brief outline of the situation.

The indicators which we have studied may be structurally represented in two dimensional space as follows:

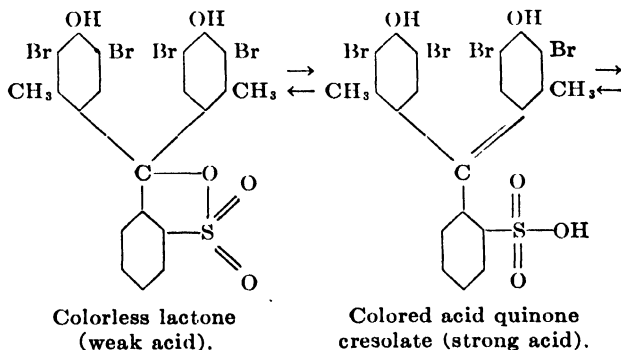


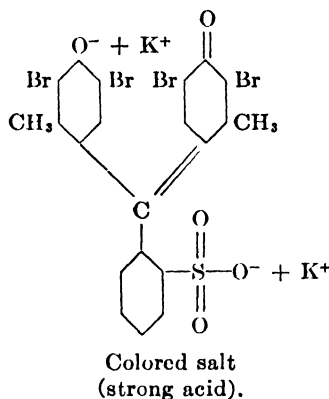
The *exact* configuration of these compounds with respect to the bromine positions is not known.

According to the original theory of Wilhelm Ostwald, indicators are weak acids or weak bases, in which the color of the undissociated form is different from that in the dissociated or ionized form. There have been some objections to this idea, among them (1) the alkali salts of phenol red are red in the solid form as well as in solution, (2) some indicator color changes show a definite time reaction (they are either ionic reactions of a higher order or more likely not ionic, but molecular reactions), and (3) Beer's law is obeyed in solutions of solvents other than water.

On the other hand, the school of Hantzsch and his collaborators has evolved the tautomeric or chromophoric theory, according to which the color of an indicator is a function of its constitution; that is, it depends on the equilibrium between a lactone form (such as colorless phenolphthalein in acid solution) and a quinone (such as red phenolphthalein in alkaline solution). However, as Kolthoff states, the structural change may be a phenomenon parallel to the color change, and yet not really be the cause of it. Also, the correlation between color and pH is not explained according to this theory without some assumption of ionization.

One may combine the two theories as Stieglitz (14) and Kolthoff have proposed. Thus, there is not only an equilibrium between the lactone and quinone forms [$L \rightleftharpoons Q$], but the quinone form will also be in equilibrium with Q^- ions and H^+ ions [$Q \rightleftharpoons Q^- + H^+$]. The ions may or may not have the same constitutions as the undissociated molecules, and the final equilibrium constant evolved, although an "apparent" equilibrium constant, yields an expression which agrees with the simple Wilhelm Ostwald form of equation. For example, brom-cresol green will behave in this manner:





The sulfonphthaleins are dibasic acids. However, the dissociation constant of the sulfonic acid group is so much stronger than that of the weak phenolic group with which the color change is mainly associated, that they may be and have been regarded as monobasic for most purposes.

In the following treatment, we may most simply consider the indicator as a weak acid, the salt or alkaline form, BA, of which is completely ionized, the acid, HA, not ionized to any significant extent. Consequently, all of the A^- anions will be derived from, and equal in concentration to BA. If the activity coefficient of the undissociated acid is assumed to be unity,

$$(1) \quad K' = \frac{K}{\gamma_{A^-}}$$

where K' denotes the apparent stoichiometric dissociation constant, K the apparent activity dissociation constant (the value of K' at infinite dilution), and γ_{A^-} the activity coefficient of the anion. If p is used to denote negative logarithm, the expression showing the relation between the hydrogen ion activity and the two forms of the indicator will then be the familiar Henderson-Hasselbalch equation:

$$(2) \quad p\alpha_{H^+} = pK' + \log \frac{[BA]}{[HA]}$$

Also, from Equation 1,

$$(3) \quad pK' = pK - p\gamma_{A^-} = pK - \Delta pK'$$

and for a dibasic acid,

$$(4) \quad pK_2' = pK_2 + p\gamma_{A-} - p\gamma_{A^{2-}} = pK_2 - \Delta pK_2'$$

The ratio $[BA]:[HA]$ may be altered by change of either α_{H^+} or of pK' . In other words, with change in pK' there is the possibility of an alteration in color of a solution with $p\alpha_{H^+}$ unchanged. When Equation 1 is applied to an indicator, BA is calculated as the colored alkali salt. In this case the pK' indicates both ionic and tautomeric equilibria. Since a constant fraction of BA is presumably tautomerized into the colored form of the indicator under any given set of conditions, this constancy may be incorporated into the general equilibrium constant K' .

That the Debye-Hückel equation makes possible an approximate quantitative calculation of the effect of neutral salts on the apparent dissociation constants of weak acids has been shown in a number of instances. Working with pure buffer solutions, Hastings and Sendroy (15) applied the Debye-Hückel theory to the study of the first and second dissociation constants of carbonic acid. Since then Cohn has studied the second dissociation constant of phosphoric acid (16) and more recently the dissociation constant of acetic acid (17). Simms (18) has recently applied the theory to the investigation of systems of weak electrolytes of organic nature.

The Debye-Hückel theory of interionic activity (19) is expressed in terms of activity coefficients, and involves the assumption of complete dissociation. When the ions are assumed to function as point charges, the Debye-Hückel equations reduce to the limiting law of Brönsted and La Mer (20).

For a single ion,

$$(5) \quad -\log \gamma_{ion} = 0.5 v^2 \sqrt{\mu}$$

where γ is the activity coefficient, v is the valence of the ion, and μ denotes the ionic strength, a unit of concentration introduced by Lewis and Randall (21). Since $\mu = \frac{1}{2} cv^2$, where c = molar or molal concentration, a molar solution of NaCl and one of $MgSO_4$ of the same strength will be as 1:1 in molar concentration, but as 1:4 in ionic strength. Where two ions are involved,

$$(6) \quad \log \frac{\gamma_1}{\gamma_2} = 0.5 (v_2^2 - v_1^2) \sqrt{\mu}$$

or for monovalent and divalent ions,

$$(7) \quad \log \frac{\gamma_1}{\gamma_2} = 1.5 \sqrt{\mu}$$

However, at higher concentrations, the ions must be regarded as finite spheres of rigid size and the Debye-Hückel equation is:

$$(8) \quad -\log \gamma_{\text{ion}} = \frac{A \nu^2 \sqrt{\mu}}{1 + B \sqrt{\mu}} \times C \mu$$

where A is a universal constant based on the absolute temperature, the dielectric constant of water, and the Boltzmann distribution coefficient; B is the composite constant involving the effective thickness of the ionic atmosphere (about $0.33 \times 10^8 \sqrt{\mu}$ cm.) and the size of the ions (usually about 2 to 5×10^{-8} cm.). C is a "salting out" term or polarization or hydration term for higher concentrations, to take account of the effect of change in dielectric constant of the environment of the particular ions whose activity is being studied. In our work, this last term can probably be neglected without causing any appreciable error, the concentrations here being no higher than $\mu = 0.2$.*

EXPERIMENTAL.

Procedure and Methods.

In order to obtain reproducible and constant potentials with the hydrogen electrode, it was necessary to buffer the solutions studied. Accordingly, phosphate and acetate mixtures of definite composition were prepared, diluted to the lowest concentrations indicated in the tables of results (M/210 acetates, and M/150 phosphates), and varying amounts of salt solutions of acetates, phosphates, NaCl, KCl, Na₂SO₄, K₂SO₄, CaCl₂, MgCl₂, and MgSO₄ were added to increase the ionic strength to about $\mu = 0.2$. The composition of all stock solutions was checked quantitatively

* It is recognized that differences in the physical and chemical constitution of solutions may cause differences in light absorption, quite apart from the effect of these differences on the ratio of the two forms of indicator as influenced by the reaction of the solution and the apparent dissociation constant of the indicator. The work of von Halban and Ebert (22), however, would seem to indicate that these effects at our concentrations of indicator and salt would be very slight, and probably negligible.

by standard methods. To study the effect, if there is any, of a non-electrolyte, some experiments were carried out in which a glucose solution was used as diluent. In order to have the concentrations of solutions the same for both electrometric and colorimetric observations, they were diluted $\frac{1}{10}$ (9 cc. + 1 cc. water) for the electrometric determinations, since the indicator addition produced the same result of dilution for the colorimetric method. The water used for all work was freshly redistilled from acid dichromate. All observations were made at $20 \pm 0.1^\circ$, colorimetric determinations being made in duplicate, electrometric until three consecutive determinations showed no greater variation than 0.2 millivolt.

In order to avoid difficulty due to the presence of minute amounts of O_2 in these dilute solutions, the extra precaution was taken of bubbling hydrogen gas through them before making the electrometric observations. The hydrogen electrode apparatus has already been described (23). The standard solution used to obtain e , the value of the saturated calomel half-cell, was 0.1 N HCl , made from Hulett's constant boiling acid. The $p\alpha_{H^+}$ was assumed to be 1.08. New platinum-coated electrodes were used for each experiment, the e being determined before and frequently after all determinations were made.

For connecting bridge, saturated KCl solution was employed. Our calculations involve the assumption that the liquid junction potential between the solution under investigation and the saturated KCl was the same in all of the solutions studied, including the 0.1 N HCl used for standardization. When we consider the different ions and ionic strengths involved in these studies, it may be questionable whether such an assumption of zero diffusion potential in each case is justified. However, the error from this source is apparently but slight in the use of the saturated KCl bridge.

The colorimetric determinations were made by adding 1 cc. of indicator (0.016 per cent brom-cresol green, 0.016 to 0.032 per cent brom-cresol purple, or 0.0075 per cent phenol red) to 9 cc. of the solution, and reading by daylight lamp against bicolor standards, as described in previous papers (24, 25). These standards depend only on the relative amounts (measured and known), of indicator in full acid and full alkaline form. Complicating factors, such as the fading of the standards, the assumption of a

pK' value for the indicator, or the consideration of the ionic strength of the standards, as in the case of phosphate and acetate buffer standards, were thereby eliminated. The concentration of indicator made according to Clark, with 1 equivalent of alkali, was only 0.00002 to 0.00006 M, not sufficient to contribute to or affect the buffering power of the solution, or its ionic strength.¹

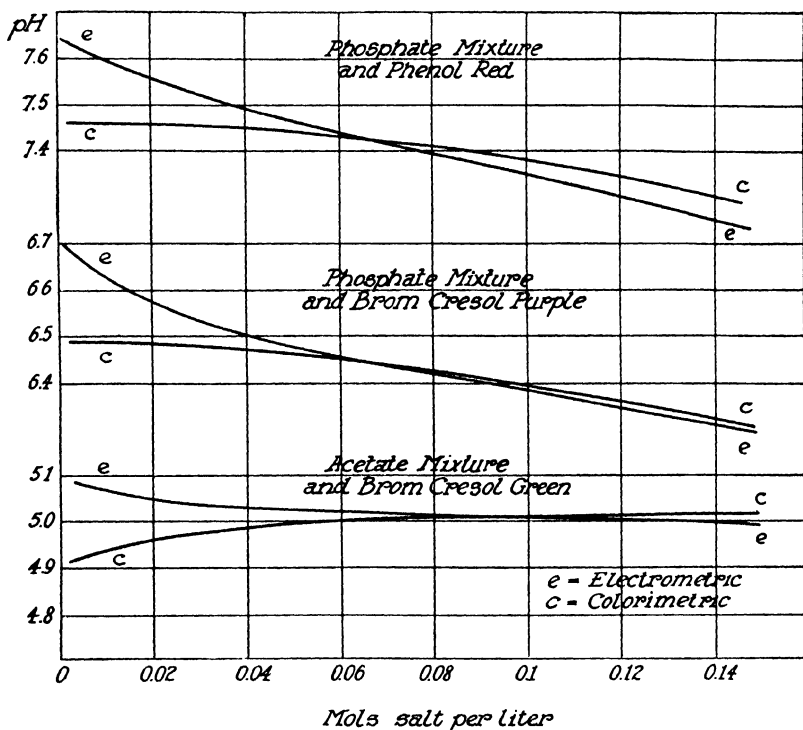


FIG. 1. Electrometric and colorimetric determinations of buffers at various dilutions.

¹ Acknowledgment should be made to the La Motte Chemical Company and to Hynson, Westcott and Dunning for supplying us with pure indicators for these experiments. No further purification was attempted by us. Several successive samples of these indicators gave identical titration curves in accordance with theory as shown by us previously (25). The titration curve of each indicator was theoretical for a solution containing a single weak acid. If other phenolphthalein derivatives had been present in significant proportions they would have measurably distorted the titra-

TABLE I.

Brom-Cresol Green pK' Change at 20° with Increase of Ionic Strength by Means of Sodium Acetate. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition.		μ	$\sqrt{\mu}$	Electro-metric p_{H^+}	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta \text{pK'}$
	$\text{HC}_2\text{H}_3\text{O}_2$	$\text{NaC}_2\text{H}_3\text{O}_2$						
	<i>M per kg. H₂O</i>	<i>M per kg. H₂O</i>						
1	0.00130	0.00303	0.00303	0.055	5.08	0.21	4.87	0.05
1 a	0.00130	0.00303	0.00303	0.055	5.08	0.23	4.85	0.07
2	0.00260	0.00606	0.00606	0.078	5.06	0.24	4.82	0.10
2 a	0.00260	0.00606	0.00606	0.078	5.08	0.24	4.84	0.08
3	0.00390	0.00909	0.00909	0.095	5.05	0.25	4.80	0.12
3 a	0.00390	0.00909	0.00909	0.095	5.06	0.25	4.81	0.11
4	0.00650	0.01515	0.01515	0.123	5.04	0.26	4.78	0.14
4 a	0.00650	0.01515	0.01515	0.123	5.06	0.26	4.80	0.12
5	0.01040	0.02424	0.02424	0.156	5.03	0.27	4.76	0.16
5 a	0.01040	0.02424	0.02424	0.156	5.04	0.26	4.78	0.14
6	0.01427	0.03333	0.03333	0.182	5.03	0.27	4.76	0.16
6 a	0.01427	0.03333	0.03333	0.182	5.03	0.27	4.76	0.16
7	0.01948	0.04545	0.04545	0.213	5.01	0.28	4.73	0.19
7 a	0.01948	0.04545	0.04545	0.213	5.02	0.29	4.73	0.19
8	0.02468	0.05757	0.05757	0.240	5.01	0.29	4.72	0.20
8 a	0.02468	0.05757	0.05757	0.240	5.02	0.30	4.72	0.20
9	0.03117	0.07272	0.07272	0.270	5.01	0.30	4.71	0.21
9 a	0.03117	0.07272	0.07272	0.270	5.02	0.31	4.71	0.21
10	0.03896	0.09090	0.09090	0.301	5.01	0.31	4.70	0.22
10 a	0.03896	0.09090	0.09090	0.301	5.01	0.32	4.69	0.23

tion curves, except in the improbable case that the impurity had the same pK' as the pure indicator. The nature of our reproducible results indicates that we were dealing with substances of a considerable degree of purity. In the case of brom-cresol green many early preparations were unusable owing to the presence of an insoluble impurity. With later preparations we had no such difficulty. These melted sharply at 215.5° (Cohen reports 217°; Cohen, B., *Pub. Health Rep., U. S. P. H. S.*, **41**, 3051 (1926)) and gave reproducible titration curves. Brom-cresol purple melted at 243°. The phenol red had no definite melting point; it decomposed at about 240°.

TABLE II.

Brom-Cresol Green pK' Change at 20° with Increase of Ionic Strength by Means of Sodium Chloride. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: acetate as in Sample 2, Table I, + NaCl.	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^{+}}$	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.00606	0.078	5.07	0.24	4.83	0.09
1 a	0.00000	0.00606	0.078	5.07	0.24	4.83	0.09
2	0.00909	0.01515	0.123	5.04	0.25	4.79	0.13
2 a	0.00909	0.01515	0.123	5.06	0.25	4.81	0.11
3	0.01818	0.02424	0.156	5.03	0.26	4.77	0.15
3 a	0.01818	0.02424	0.156	5.03	0.26	4.77	0.15
4	0.02727	0.03333	0.182	5.01	0.27	4.74	0.18
4 a	0.02727	0.03333	0.182	5.01	0.27	4.74	0.18
5	0.04545	0.05151	0.227	5.00	0.28	4.72	0.20
5 a	0.04545	0.05151	0.227	5.00	0.28	4.72	0.20
6	0.06363	0.06969	0.264	4.98	0.30	4.68	0.24
6 a	0.06363	0.06969	0.264	4.99	0.30	4.69	0.23
7	0.08181	0.08787	0.296	4.97	0.31	4.66	0.26
7 a	0.08181	0.08787	0.296	4.97	0.31	4.66	0.26
8	0.10908	0.11514	0.339	4.96	0.32	4.64	0.28
8 a	0.10908	0.11514	0.339	4.97	0.33	4.64	0.28
9	0.13635	0.14241	0.377	4.94	0.33	4.61	0.31
9 a	0.13635	0.14241	0.377	4.96	0.34	4.62	0.30
10	0.16362	0.16968	0.412	4.93	0.34	4.59	0.33
10 a	0.16362	0.16968	0.412	4.95	0.35	4.60	0.32
11	0.21210	0.21816	0.467	4.93	0.36	4.57	0.35

RESULTS.

When a M/15 phosphate solution of about $p\alpha_{H^{+}} = 7.4$, such as is used for color standards, is diluted in several steps to 30 times its

TABLE III.

Brom-Cresol Green pK' Change at 20° with Increase of Ionic Strength by Means of Potassium Chloride. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: acetate as in Sample 2, Table I, + KCl.	μ	$\sqrt{\mu}$	Electro-metric $p\alpha_{H^{+}}$	Colorimetric color ratio: $\log R$.	pK' indicator.	ΔpK
	<i>M per kg. H₂O</i>						
1	0.00000	0.00606	0.078	5.07	0.22	4.85	0.07
1 a	0.00000	0.00606	0.078	5.09	0.24	4.85	0.07
1 b	0.00000	0.00606	0.078	5.07	0.24	4.83	0.09
2	0.00909	0.01515	0.123	5.06	0.24	4.82	0.10
2 a	0.00909	0.01515	0.123	5.04	0.25	4.79	0.13
3	0.01818	0.02424	0.156	5.04	0.27	4.77	0.15
3 a	0.01818	0.02424	0.156	5.03	0.26	4.77	0.15
4	0.02727	0.03333	0.182	5.03	0.29	4.74	0.18
4 a	0.03030	0.03636	0.191	5.02	0.29	4.73	0.19
5	0.04545	0.05151	0.227	5.02	0.30	4.72	0.20
5 a	0.04545	0.05151	0.227	5.01	0.31	4.70	0.22
6	0.06363	0.06969	0.264	5.01	0.31	4.70	0.22
6 a	0.06363	0.06969	0.264	4.99	0.32	4.67	0.25
6 b	0.06363	0.06969	0.264	5.00	0.32	4.68	0.24
7	0.08181	0.08787	0.296	5.00	0.34	4.66	0.26
7 a	0.08181	0.08787	0.296	5.00	0.34	4.66	0.26
8	0.10908	0.11514	0.339	4.99	0.34	4.65	0.27
8 a	0.10908	0.11514	0.339	4.99	0.35	4.64	0.28
8 b	0.10908	0.11514	0.339	4.98	0.35	4.63	0.29
9	0.13635	0.14241	0.377	4.98	0.35	4.63	0.29
9 a	0.13635	0.14241	0.377	4.98	0.35	4.63	0.29
9 b	0.13635	0.14241	0.377	4.98	0.36	4.62	0.30
10	0.16362	0.16968	0.412	4.98	0.35	4.63	0.29
10 a	0.16362	0.16968	0.412	4.97	0.36	4.61	0.31
10 b	0.16362	0.16968	0.412	4.98	0.36	4.62	0.30
11	0.19998	0.20604	0.460	4.97	0.36	4.61	0.31
11 a	0.19998	0.20604	0.460	4.97	0.36	4.61	0.31
11 b	0.19998	0.20604	0.460	4.96	0.37	4.59	0.33

TABLE IV.

Brom-Cresol Green pK' Change at 20° with Increase of Ionic Strength by Means of Sodium Sulfate. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: acetate as in Sample 2, Table I, + Na ₂ SO ₄ .	μ	$\sqrt{\mu}$	Electrometric p_{H^+}	Colorimetric color ratio: log R.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.00606	0.078	5.06	0.24	4.82	0.10
1 a	0.00000	0.00606	0.078	5.09	0.24	4.85	0.07
2	0.00303	0.01515	0.123	5.06	0.25	4.81	0.11
2 a	0.00303	0.01515	0.123	5.06	0.25	4.81	0.11
3	0.00606	0.02424	0.156	5.05	0.26	4.79	0.13
3 a	0.00606	0.02424	0.156	5.03	0.26	4.77	0.15
4	0.00909	0.03333	0.182	5.04	0.27	4.77	0.15
4 a	0.00909	0.03333	0.182	5.03	0.27	4.76	0.16
5	0.01515	0.05151	0.227	5.01	0.28	4.73	0.19
5 a	0.01515	0.05151	0.227	5.03	0.29	4.74	0.18
6	0.02424	0.06969	0.264	5.01	0.30	4.71	0.21
6 a	0.02424	0.06969	0.264	5.00	0.30	4.70	0.22
7	0.02727	0.08787	0.296	5.00	0.31	4.69	0.23
7 a	0.02727	0.08787	0.296	4.99	0.31	4.68	0.24
8	0.03636	0.11514	0.339	4.99	0.32	4.67	0.25
8 a	0.03636	0.11514	0.339	4.99	0.32	4.67	0.25
9	0.04545	0.14241	0.377	4.98	0.33	4.65	0.27
9 a	0.04545	0.14241	0.377	4.98	0.33	4.65	0.27
10	0.05454	0.16968	0.412	4.98	0.34	4.64	0.28
10 a	0.05454	0.16968	0.412	4.97	0.34	4.63	0.29
11	0.06666	0.20604	0.454	4.95	0.35	4.60	0.32
11 a	0.07070	0.21816	0.467	4.96	0.35	4.61	0.31
12	0.08060	0.24786	0.498	4.94	0.36	4.58	0.34

TABLE V.

Brom-Cresol Green pK' Change at 20° with Increase of Ionic Strength by Means of Potassium Sulfate. Colorimetric Readings with Indicator Concentration = 0.0002 M.

Sample No.	Composition: acetate as in Sample 2, Table I, + K ₂ SO ₄ .	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^{+}}$	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.00606	0.078	5.07	0.24	4.83	0.09
1 a	0.00000	0.00606	0.078	5.07	0.24	4.83	0.09
2	0.00228	0.01288	0.113	5.07	0.25	4.82	0.10
2 a	0.00228	0.01288	0.113	5.07	0.25	4.82	0.10
3	0.00455	0.01969	0.140	5.06	0.25	4.81	0.11
3 a	0.00455	0.01969	0.140	5.06	0.26	4.80	0.12
4	0.00682	0.02652	0.163	5.05	0.26	4.79	0.13
4 a	0.00682	0.02652	0.163	5.05	0.27	4.78	0.14
5	0.01137	0.04015	0.203	5.04	0.27	4.77	0.15
5 a	0.01137	0.04015	0.203	5.04	0.28	4.76	0.16
6	0.01591	0.05379	0.232	5.03	0.29	4.74	0.18
6 a	0.01591	0.05379	0.232	5.02	0.30	4.72	0.20
7	0.02046	0.06742	0.260	5.02	0.30	4.72	0.20
7 a	0.02046	0.06742	0.260	5.02	0.31	4.71	0.21
8	0.02727	0.08787	0.296	5.01	0.32	4.69	0.23
8 a	0.02727	0.08787	0.296	5.00	0.32	4.68	0.24
9	0.03409	0.10833	0.329	5.01	0.34	4.67	0.25
9 a	0.03409	0.10833	0.329	5.00	0.33	4.67	0.25
10	0.04091	0.12879	0.359	5.00	0.34	4.66	0.24
10 a	0.04091	0.12879	0.359	5.00	0.34	4.66	0.24
11	0.05303	0.16514	0.406	4.98	0.35	4.63	0.21

original volume, and the colorimetric $p\alpha_{H^{+}}$ is read against the original M/15 standards at each dilution, with indicator concentrations constant, the result obtained with phenol red is such as is

TABLE VI.

Brom-Cresol Green pK' Change at 20° with Increase of Ionic Strength by Means of Calcium Chloride. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: acetate as in Sample 2, Table I, + CaCl ₂ .	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>m per kg. H₂O</i>						
1	0.00000	0.00604	0.078	5.08	0.24	4.84	0.08
1 a	0.00000	0.00604	0.078	5.09	0.24	4.85	0.07
2	0.00312	0.01540	0.124	5.05	0.25	4.80	0.12
2 a	0.00312	0.01540	0.124	5.04	0.26	4.78	0.14
3	0.00624	0.02478	0.157	5.03	0.27	4.76	0.16
3 a	0.00624	0.02478	0.157	5.04	0.28	4.76	0.16
4	0.00937	0.03414	0.185	5.01	0.29	4.72	0.20
4 a	0.01025	0.03679	0.192	5.01	0.30	4.71	0.21
5	0.01561	0.05287	0.230	4.99	0.31	4.68	0.24
5 a	0.01538	0.05218	0.228	5.01	0.31	4.70	0.22
6	0.02186	0.07160	0.267	4.98	0.33	4.65	0.27
6 a	0.02153	0.07063	0.266	4.96	0.32	4.64	0.28
7	0.02810	0.09034	0.300	4.96	0.34	4.62	0.30
7 a	0.02769	0.08910	0.298	4.96	0.33	4.63	0.29
8	0.03747	0.11844	0.344	4.95	0.35	4.60	0.32
8 a	0.03691	0.11677	0.341	4.93	0.33	4.60	0.32
9	0.04684	0.14654	0.382	4.92	0.35	4.57	0.35
9 a	0.04614	0.14445	0.380	4.92	0.34	4.58	0.34
10	0.05620	0.17464	0.417	4.91	0.35	4.56	0.36
10 a	0.05536	0.17212	0.415	4.90	0.34	4.56	0.36
11	0.07286	0.22460	0.473	4.88	0.36	4.52	0.40
11 a	0.07177	0.22135	0.470	4.89	0.35	4.54	0.38

shown in Fig. 1. The apparent slight $p\alpha_{H^+}$ change is in the direction of an increase with dilution. The same procedure with a

TABLE VII.

Brom-Cresol Green pK' Change at 20° with Increase of Ionic Strength by Means of Magnesium Chloride. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: acetate as in Sample 2, Table I, + MgCl ₂ .	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg H₂O</i>						
1	0.00000	0.00604	0.078	5.07	0.24	4.83	0.09
1 a	0.00000	0.00604	0.078	5.07	0.24	4.83	0.09
2	0.00327	0.01585	0.126	5.03	0.26	4.77	0.15
2 a	0.00327	0.01585	0.126	5.03	0.26	4.77	0.15
3	0.00655	0.02568	0.160	5.02	0.28	4.74	0.18
3 a	0.00655	0.02568	0.160	5.01	0.29	4.72	0.20
4	0.00982	0.03549	0.188	5.00	0.30	4.70	0.22
4 a	0.01090	0.03875	0.197	5.00	0.30	4.70	0.22
5	0.01527	0.05185	0.228	4.97	0.31	4.66	0.26
5 a	0.01527	0.05185	0.228	4.97	0.31	4.66	0.26
6	0.02181	0.07147	0.267	4.95	0.32	4.63	0.29
6 a	0.02181	0.07147	0.267	4.96	0.32	4.64	0.28
7	0.02836	0.09110	0.302	4.94	0.33	4.61	0.31
7 a	0.02836	0.09110	0.302	4.95	0.32	4.63	0.29
8	0.03708	0.11728	0.343	4.92	0.34	4.58	0.34
8 a	0.03708	0.11728	0.343	4.93	0.33	4.60	0.32
9	0.04580	0.14345	0.379	4.90	0.34	4.56	0.36
9 a	0.04580	0.14345	0.379	4.91	0.34	4.57	0.35
10	0.05672	0.17618	0.420	4.88	0.35	4.53	0.39
10 a	0.05672	0.17618	0.420	4.90	0.34	4.56	0.36
11	0.07198	0.22198	0.471	4.86	0.35	4.51	0.41
11 a	0.07198	0.22198	0.471	4.87	0.35	4.52	0.40

M/15 phosphate mixture of $p\alpha_{H^+}$ about 6.4 and brom-cresol purple yields a similar result. On the other hand, with a M/7 acetate mixture of $p\alpha_{H^+}$ about 5.0 and brom-cresol green, the curve ob-

tained indicates, with reference to the original standard, a $p\alpha_{H^+}$ decreasing with dilution.

An inspection of the *electrometric* dilution curves shows that there is no essential difference in the behavior of the phosphates and acetates, but that the above outlined difference is due to the fact that the buffer salts have other, though lesser, effects on the indicators than the effect exerted through $p\alpha_{H^+}$. In each case, dilution of the buffer results in an *increase* of the actual $p\alpha_{H^+}$ determined electrometrically. The colorimetric and electrometric observations agree at one point only; namely, at the concentration of salt corresponding to that of the colorimetric stand-

TABLE VIII.

Brom-Cresol Green pK' at 20° with Addition of Glucose. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: acetate as in Sample 2, Table I, + glucose.	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per l.</i>						
1	0.0000	0.00604	0.078	5.07	0.24	4.83	0.09
2	0.0111	0.00604	0.078	5.08	0.25	4.83	0.09
3	0.0222	0.00604	0.078	5.07	0.24	4.83	0.09
4	0.0333	0.00604	0.078	5.07	0.25	4.82	0.10
5	0.0555	0.00604	0.078	5.07	0.24	4.83	0.09
6	0.0888	0.00604	0.078	5.06	0.25	4.81	0.11
7	0.1333	0.00604	0.078	5.07	0.24	4.83	0.09
8	0.2000	0.00604	0.078	5.05	0.24	4.81	0.11

ards used for the color readings. Increased concentration again causes a divergence of the two curves, but the discrepancy is of a different sign. Thus the relative salt concentrations of the unknown material and the color standard determine the magnitude and the algebraic sign of what is known in the literature as "salt error." In the sense in which it is used in this paper, this is the difference between the colorimetric and the electrometric readings: $p\alpha_{H^+}(c) - p\alpha_{H^+}(e)$.

The experimental data and the results obtained are given in Tables I to XXIII inclusive. The values of pK' of the indicators were calculated according to Equation 2 from the electrometric

TABLE IX.

Brom-Cresol Purple pK' Change at 20° with Increase of Ionic Strength by Means of Sodium and Potassium Phosphates. Colorimetric Readings with Indicator Concentration = 0.00003 M.

Sample No.	Composition.		μ	$\sqrt{\mu}$	Electro-metric $p\alpha H^{+}$.	Colorimetric color ratio: log R.	pK' indicator.	$\Delta pK'$
	KH ₂ PO ₄	Na ₂ HPO ₄						
	<i>M per kg. H₂O</i>	<i>M per kg. H₂O</i>						
1	0.00144	0.00057	0.00314	0.056	6.69	0.30	6.39	0.07
1 a	0.00144	0.00057	0.00314	0.056	6.69	0.30	6.39	0.07
2	0.00288	0.00113	0.00628	0.079	6.68	0.29	6.39	0.07
2 a	0.00288	0.00113	0.00628	0.079	6.67	0.30	6.37	0.09
3	0.00433	0.00170	0.00943	0.097	6.66	0.29	6.37	0.09
3 a	0.00433	0.00170	0.00943	0.097	6.65	0.29	6.36	0.10
4	0.00576	0.00227	0.01256	0.112	6.64	0.28	6.36	0.10
4 a	0.00576	0.00227	0.01256	0.112	6.64	0.30	6.34	0.12
5	0.00866	0.00340	0.01886	0.137	6.61	0.28	6.33	0.13
5 a	0.00866	0.00340	0.01886	0.137	6.58	0.29	6.29	0.17
6	0.01299	0.00510	0.02829	0.168	6.57	0.27	6.30	0.16
6 a	0.01299	0.00510	0.02829	0.168	6.58	0.30	6.28	0.18
7	0.01732	0.00680	0.03772	0.194	6.55	0.27	6.28	0.18
7 a	0.01732	0.00680	0.03772	0.194	6.56	0.29	6.27	0.19
8	0.02165	0.00850	0.04716	0.217	6.52	0.27	6.25	0.21
8 a	0.02165	0.00850	0.04716	0.217	6.53	0.28	6.25	0.21
9	0.02598	0.01020	0.05658	0.238	6.50	0.26	6.24	0.22
9 a	0.02598	0.01020	0.05658	0.238	6.51	0.27	6.24	0.22
10	0.03174	0.01247	0.06913	0.263	6.48	0.26	6.22	0.24
10 a	0.03174	0.01247	0.06913	0.263	6.49	0.26	6.23	0.23
11	0.03754	0.01475	0.07677	0.277	6.46	0.25	6.21	0.25
11 a	0.03754	0.01475	0.07677	0.277	6.46	0.24	6.22	0.24
12	0.04330	0.01700	0.09431	0.307	6.44	0.23	6.21	0.25
12 a	0.04330	0.01700	0.09431	0.307	6.44	0.23	6.21	0.25

TABLE X.

Brom-Cresol Purple pK' Change at 20° with Increase of Ionic Strength by Means of Sodium Chloride. Colorimetric Readings with Indicator Concentration = 0.00003 M.

Sample No.	Composition: phosphate as in Sample 3, Table IX, + NaCl.	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.00948	0.097	6.65	0.28	6.37	0.09
1 a	0.00000	0.00948	0.097	6.60	0.29	6.31	0.15
2	0.00914	0.01862	0.136	6.59	0.28	6.31	0.15
2 a	0.00914	0.01862	0.136	6.58	0.28	6.30	0.16
3	0.01828	0.02776	0.167	6.56	0.28	6.28	0.18
3 a	0.01828	0.02776	0.167	6.53	0.28	6.25	0.21
4	0.02742	0.03690	0.192	6.52	0.27	6.25	0.21
4 a	0.02742	0.03690	0.192	6.51	0.27	6.24	0.22
5	0.04570	0.05518	0.235	6.48	0.26	6.22	0.24
5 a	0.04570	0.05518	0.235	6.46	0.26	6.20	0.26
6	0.06398	0.07346	0.271	6.43	0.24	6.19	0.27
6 a	0.06398	0.07346	0.271	6.42	0.24	6.18	0.28
7	0.08226	0.09174	0.303	6.38	0.22	6.16	0.30
7 a	0.08226	0.09174	0.303	6.38	0.22	6.16	0.30
8	0.10968	0.11916	0.345	6.34	0.20	6.14	0.32
8 a	0.10968	0.11916	0.345	6.35	0.21	6.14	0.32
9	0.13710	0.14658	0.383	6.31	0.18	6.13	0.33
9 a	0.13710	0.14658	0.383	6.31	0.19	6.12	0.34
10	0.16452	0.17400	0.417	6.28	0.15	6.13	0.33
10 a	0.16452	0.17400	0.417	6.29	0.17	6.12	0.34

$p\alpha_{H^+}$ and the colorimetric readings of the samples against bicolor standards. The standards were marked in terms of the logarithm of the ratio of the alkaline to the acid form of the indicator. The values of pK' so calculated involve the experimental errors in determination of $p\alpha_{H^+}$ electrometrically and colorimetrically, which

TABLE XI.

Brom-Cresol Purple pK' Change at 20° with Increase of Ionic Strength by Means of Potassium Chloride. Colorimetric Readings with Indicator Concentration = 0.00003 M.

Sample No.	Composition: phosphate as in Sample 3, Table IX, + KCl.	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.00948	0.097	6.64	0.28	6.36	0.10
1 a	0.00000	0.00948	0.097	6.65	0.29	6.36	0.10
2	0.00910	0.01858	0.136	6.58	0.27	6.31	0.15
2 a	0.00910	0.01858	0.136	6.59	0.28	6.31	0.15
3	0.01820	0.02768	0.166	6.56	0.27	6.29	0.17
3 a	0.01820	0.02768	0.166	6.56	0.28	6.28	0.18
4	0.02730	0.03678	0.192	6.52	0.27	6.25	0.21
4 a	0.02730	0.03678	0.192	6.53	0.27	6.26	0.20
5	0.04550	0.05498	0.234	6.49	0.26	6.23	0.23
5 a	0.04550	0.05498	0.234	6.48	0.26	6.22	0.24
6	0.06370	0.07318	0.270	6.44	0.24	6.20	0.26
6 a	0.06370	0.07318	0.270	6.44	0.25	6.19	0.27
7	0.08190	0.09138	0.302	6.42	0.23	6.19	0.27
7 a	0.08190	0.09138	0.302	6.42	0.24	6.18	0.28
8	0.10920	0.11868	0.344	6.37	0.21	6.16	0.30
8 a	0.10920	0.11868	0.344	6.38	0.23	6.15	0.31
9	0.13650	0.14598	0.382	6.35	0.22	6.13	0.33
10	0.16380	0.17328	0.416	6.32	0.18	6.14	0.32
10 a	0.16380	0.17328	0.416	6.32	0.20	6.12	0.34

may be 0.01 unit in each case, or even 0.02 for colorimetric observations. The change in pK' with variation of ionic strength was then plotted against $\sqrt{\mu}$, and the curve drawn through the points was extrapolated to zero ionic strength. This was done for each salt used to vary the ionic strength. The results are

TABLE XII.

Brom-Cresol Purple pK' Change with Increase of Ionic Strength by Means of Sodium Sulfate. Colorimetric Readings with Indicator Concentration = 0.00003 M.

Sample No.	Composition: phosphate as in Sample 3, Table IX, + Na ₂ SO ₄ .	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg H₂O</i>						
1	0.00000	0.00946	0.097	6.65	0.29	6.36	0.10
1 a	0.00000	0.00946	0.097	6.66	0.29	6.37	0.09
2	0.00270	0.01754	0.132	6.61	0.29	6.32	0.14
2 a	0.00270	0.01754	0.132	6.61	0.28	6.33	0.13
3	0.00539	0.02563	0.160	6.57	0.28	6.29	0.17
3 a	0.00539	0.02563	0.160	6.57	0.28	6.29	0.17
4	0.00809	0.03371	0.184	6.55	0.27	6.28	0.18
4 a	0.00809	0.03371	0.184	6.55	0.27	6.28	0.18
5	0.01348	0.04988	0.223	6.50	0.26	6.24	0.22
5 a	0.01348	0.04988	0.223	6.51	0.27	6.24	0.22
6	0.01887	0.06605	0.257	6.47	0.26	6.21	0.25
6 a	0.01887	0.06605	0.257	6.48	0.27	6.21	0.25
7	0.02426	0.08222	0.287	6.46	0.27	6.19	0.27
7 a	0.02426	0.08222	0.287	6.45	0.26	6.19	0.27
8	0.03234	0.10548	0.325	6.42	0.25	6.17	0.29
8 a	0.03234	0.10548	0.325	6.42	0.25	6.17	0.29
9	0.04043	0.13073	0.361	6.39	0.23	6.16	0.30
9 a	0.04043	0.13073	0.361	6.38	0.23	6.15	0.31
10	0.04851	0.15499	0.393	6.36	0.22	6.14	0.32
10 a	0.04851	0.15499	0.393	6.36	0.22	6.14	0.32

given in Table XXIV. Considering the uncertainty attached to such extrapolations from even the most dilute solutions with which these experiments were performed, the agreement of pK for the different solutions is fairly good. The average pK values thus obtained were then used as the basis for calculating $\Delta pK'$.

TABLE XIII.

Brom-Cresol Purple pK' Change with Increase of Ionic Strength by Means of Potassium Sulfate. Colorimetric Readings with Indicator Concentration = 0.00003 M.

Sample No.	Composition: phosphate as in Sample 3, Table IX, + K ₂ SO ₄ .	μ	$\sqrt{\mu}$	Electrometric p α_{II}^{+} .	Colorimetric color ratio: log R.	pK' indicator.	Δ pK'
	<i>M per kg. H₂O</i>						
1	0.00000	0.00948	0.097	6.66	0.30	6.36	0.10
1 a	0.00000	0.00948	0.097	6.66	0.30	6.36	0.10
2	0.00227	0.01629	0.127	6.62	0.30	6.32	0.14
2 a	0.00227	0.01629	0.127	6.62	0.29	6.33	0.13
3	0.00454	0.02310	0.152	6.60	0.29	6.31	0.15
3 a	0.00454	0.02310	0.152	6.60	0.29	6.31	0.15
4	0.00681	0.02991	0.173	6.57	0.28	6.29	0.17
4 a	0.00681	0.02991	0.173	6.57	0.28	6.29	0.17
5	0.01135	0.04353	0.208	6.54	0.28	6.26	0.20
5 a	0.01135	0.04353	0.208	6.56	0.27	6.29	0.17
6	0.01589	0.05715	0.239	6.51	0.27	6.24	0.22
6 a	0.01589	0.05715	0.239	6.53	0.27	6.26	0.20
7	0.02043	0.07077	0.266	6.49	0.26	6.23	0.23
7 a	0.02043	0.07077	0.266	6.49	0.26	6.23	0.23
8	0.02724	0.09120	0.302	6.46	0.25	6.21	0.25
8 a	0.02724	0.09120	0.302	6.46	0.25	6.21	0.25
9	0.03905	0.11163	0.334	6.42	0.23	6.19	0.27
9 a	0.03905	0.11163	0.334	6.44	0.24	6.20	0.26
10	0.04086	0.13206	0.363	6.39	0.22	6.17	0.29
10 a	0.04086	0.13206	0.363	6.41	0.23	6.18	0.28
11	0.04697	0.15038	0.388	6.40	0.22	6.18	0.28
12	0.05297	0.16838	0.410	6.38	0.21	6.17	0.29

TABLE XIV.

Brom-Cresol Purple pK' Change with Increase of Ionic Strength by Means of Magnesium Chloride. Colorimetric Readings with Indicator Concentration = 0.00006 M.

Sample No.	Composition: phosphate as in Sample 3, Table IX, + MgCl ₂ .	μ	$\sqrt{\mu}$	Electro-metric p_{aH^+} .	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.00948	0.097	6.63	0.28	6.35	0.11
1 a	0.00000	0.00948	0.097	6.64	0.28	6.36	0.10
2	0.00329	0.01934	0.139	6.44	0.13	6.31	0.15
2 a	0.00329	0.01934	0.139	6.44	0.12	6.32	0.14
3	0.00657	0.02918	0.171	6.30	0.04	6.26	0.20
3 a	0.00657	0.02918	0.171	6.31	0.03	6.28	0.18
4	0.01094	0.04230	0.206	6.19	-0.05	6.24	0.22
4 a	0.01094	0.04230	0.206	6.19	-0.07	6.26	0.20
5	0.01641	0.05871	0.242	6.09	-0.13	6.22	0.24
5 a	0.01641	0.05871	0.242	6.10	-0.15	6.25	0.21
6	0.02298	0.07840	0.280	6.01	-0.18	6.19	0.27
6 a	0.02298	0.07840	0.280	6.01	-0.18	6.19	0.27
7	0.02954	0.09810	0.313	5.92	-0.22	6.14	0.32
7 a	0.02954	0.09810	0.313	5.95	-0.23	6.18	0.28
8	0.03939	0.12764	0.357	5.85	-0.26	6.11	0.35
8 a	0.03939	0.12764	0.357	5.87	-0.27	6.14	0.32
9	0.04923	0.15715	0.396	5.80	-0.30	6.10	0.36
9 a	0.04923	0.15715	0.396	5.81	-0.30	6.11	0.35
10	0.05908	0.18670	0.432	5.75	-0.35	6.10	0.36
10 a	0.05908	0.18670	0.432	5.75	-0.33	6.08	0.38

In Figs. 2 to 4, curves were drawn through the experimental points determined for each salt, and all were extrapolated to the same average pK value.

TABLE XV.

Brom-Cresol Purple pK' Change with Increase of Ionic Strength by Means of Magnesium Sulfate. Colorimetric Readings with Indicator Concentration = 0.00006 M.

Sample No.	Composition: phosphate as in Sample 3, Table IX, + MgSO ₄ .	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.00948	0.097	6.65	0.28	6.37	0.09
1 a	0.00000	0.00948	0.097	6.63	0.27	6.36	0.10
2	0.00227	0.01857	0.136	6.51	0.17	6.34	0.12
2 a	0.00227	0.01857	0.136	6.51	0.16	6.35	0.11
3	0.00455	0.02766	0.166	6.41	0.10	6.31	0.15
3 a	0.00455	0.02766	0.166	6.41	0.09	6.32	0.14
4	0.00758	0.03978	0.199	6.32	0.03	6.29	0.17
4 a	0.00758	0.03978	0.199	6.32	0.03	6.29	0.17
5	0.01136	0.05493	0.234	6.25	-0.04	6.29	0.17
5 a	0.01136	0.05493	0.234	6.25	-0.02	6.27	0.19
6	0.01591	0.07311	0.270	6.18	-0.08	6.26	0.20
6 a	0.01591	0.07311	0.270	6.18	-0.07	6.25	0.21
7	0.02045	0.09130	0.302	6.13	-0.11	6.24	0.22
7 a	0.02045	0.09130	0.302	6.13	-0.12	6.25	0.21
8	0.02727	0.11856	0.344	6.06	-0.15	6.21	0.25
8 a	0.02727	0.11856	0.344	6.07	-0.15	6.22	0.24
9	0.03409	0.14584	0.382	6.02	-0.18	6.20	0.26
9 a	0.03409	0.14584	0.382	6.02	-0.17	6.18	0.28
10	0.04091	0.17312	0.416	5.97	-0.21	6.18	0.28
10 a	0.04091	0.17312	0.416	5.97	-0.20	6.17	0.29

DISCUSSION.

A search of the literature revealed only one paper containing data which could be used in estimating the pK' variation of these indicators at different ionic strengths. Lepper and Martin (26)

studied the "salt error" of phenol red at 18°. They diluted phosphate and bicarbonate buffers with water and NaCl solutions, and determined $p\alpha_{H^+}$ electrometrically and colorimetrically. Their colorimetric solutions were diluted 10 per cent, whereas the electrometric samples were not. On the assumption that pK' of phenol red = 7.78 for the phosphate color standards they used, the pK' values at the different ionic strengths have been calculated from their Tables I to V, and are given in our Table XXV. Fig. 5 indicates the correspondence of the two sets of studies. The agreement is good at higher ionic strengths, but Løpper and

TABLE XVI.

Brom-Cresol Purple pK' at 20° with Addition of Glucose. Colorimetric Readings with Indicator Concentration = 0.00006 M.

Sample No.	Composition, phosphate as in Sample 4, Table IX, + glucose.	μ	$\sqrt{\mu}$	Electrometric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator	$\Delta pK'$
	<i>M per l.</i>						
1	0.0000	0.01256	0.112	6.61	0.27	6.34	0.12
2	0.0111	0.01256	0.112	6.62	0.27	6.35	0.11
3	0.0222	0.01256	0.112	6.61	0.27	6.34	0.12
4	0.0333	0.01256	0.112	6.60	0.26	6.34	0.12
5	0.0555	0.01256	0.112	6.58	0.27	6.31	0.15
6	0.0888	0.01256	0.112	6.59	0.25	6.34	0.12
7	0.1333	0.01256	0.112	6.56	0.25	6.31	0.15
8	0.2000	0.01256	0.112	6.55	0.23	6.32	0.14

Martin's results for phosphate mixtures alone do not fit in well with the rest of the data.

Unit of Electrolyte Concentration.—The ionic strength principle enunciated by Lewis and Randall (21) is to the effect that *in dilute solutions*, the activity coefficient of an ion is a function of the total ionic strength only. This principle has been questioned by Brønsted and La Mer (20) and more recently by La Mer and his collaborators (27, 28) for salts involving symmetrical and unsymmetrical high valence types, but in general it seems to hold fairly well for uni-univalent salts even in higher concentrations than 0.001 M. The ionic strength μ is a unit of concentration which takes into account the number and valence of the ions present

TABLE XVII

Phenol Red pK' Change at 20° with Increase of Ionic Strength by Means of Sodium and Potassium Phosphates Colorimetric Readings with Indicator Concentration = 0.0002 M

Sample No	Composition		μ	$\sqrt{\mu}$	Electrometric $p\alpha H^+$	Colorimetric color ratio $\log R$	pK' indicator	$\Delta pK'$
	KH ₂ PO ₄	Na ₂ HPO ₄						
	<i>M per kg H₂O</i>	<i>M per kg H₂O</i>						
1	0.00039	0.00163	0.00527	0.073	7.64	-0.35	7.99	0.05
1 a	0.00039	0.00163	0.00527	0.073	7.63	-0.35	7.98	0.06
1 b	0.00056	0.00145	0.00491	0.070	7.43	-0.53	7.96	0.08
2	0.00078	0.00325	0.01053	0.103	7.62	-0.33	7.95	0.09
2 a	0.00078	0.00325	0.01053	0.103	7.63	-0.33	7.96	0.08
2 b	0.00078	0.00325	0.01053	0.103	7.65	-0.32	7.97	0.07
2 c	0.00112	0.00290	0.00982	0.099	7.45	-0.53	7.98	0.06
3	0.00116	0.00488	0.01579	0.126	7.61	-0.32	7.93	0.11
3 a	0.00116	0.00488	0.01579	0.126	7.63	-0.33	7.96	0.08
3 b	0.00169	0.00435	0.01472	0.121	7.44	-0.53	7.97	0.07
4	0.00193	0.00813	0.02632	0.162	7.61	-0.34	7.95	0.09
4 a	0.00155	0.00650	0.02107	0.145	7.59	-0.34	7.93	0.11
4 b	0.00155	0.00650	0.02107	0.145	7.60	-0.32	7.92	0.12
4 c	0.00224	0.00580	0.01964	0.140	7.43	-0.53	7.96	0.08
5	0.00232	0.00976	0.03159	0.178	7.58	-0.34	7.92	0.12
5 a	0.00232	0.00976	0.03159	0.178	7.58	-0.33	7.91	0.13
5 b	0.00232	0.00976	0.03159	0.178	7.59	-0.34	7.93	0.11
5 c	0.00336	0.00870	0.02944	0.172	7.39	-0.54	7.93	0.11
6	0.00402	0.01690	0.05474	0.234	7.55	-0.35	7.90	0.14
6 a	0.00348	0.01464	0.04740	0.218	7.55	-0.34	7.89	0.15
6 b	0.00348	0.01464	0.04740	0.218	7.56	-0.34	7.90	0.14
6 c	0.00504	0.01304	0.04416	0.210	7.37	-0.55	7.92	0.12
7	0.00502	0.02113	0.06842	0.261	7.54	-0.36	7.90	0.14
7 a	0.00464	0.01957	0.06333	0.252	7.52	-0.35	7.87	0.17
7 b	0.00464	0.01957	0.06333	0.252	7.52	-0.35	7.87	0.17
7 c	0.00464	0.01957	0.06333	0.252	7.54	-0.35	7.89	0.15
7 d	0.00672	0.01740	0.05888	0.243	7.33	-0.56	7.89	0.15

TABLE XVII—*Concluded.*

Sample No.	Composition.		μ	$\sqrt{\mu}$	Electro-metric $p\alpha H^+$.	Colori-metric color ratio: log <i>R</i> .	pK' indica-tor.	Δ pK'
	KH ₂ PO ₄	Na ₂ HPO ₄						
	<i>M per kg H₂O</i>	<i>M per kg H₂O</i>						
8	0.00579	0.02439	0.07895	0.281	7.51	-0.36	7.87	0.17
8 a	0.00579	0.02439	0.07895	0.281	7.50	-0.36	7.86	0.18
8 b	0.00579	0.02439	0.07895	0.281	7.51	-0.35	7.86	0.18
8 c	0.00579	0.02439	0.07895	0.281	7.52	-0.36	7.88	0.16
8 d	0.00840	0.02175	0.07360	0.271	7.31	-0.56	7.87	0.17
9	0.00696	0.02927	0.09475	0.308	7.50	-0.37	7.87	0.17
9 a	0.00696	0.02927	0.09475	0.308	7.48	-0.37	7.85	0.19
9 b	0.00696	0.02927	0.09475	0.308	7.49	-0.36	7.85	0.19
9 c	0.00696	0.02927	0.09475	0.308	7.48	-0.37	7.85	0.19
9 d	0.01008	0.02610	0.08832	0.297	7.26	-0.57	7.83	0.21
10	0.00804	0.03380	0.10946	0.331	7.47	-0.37	7.84	0.20
10 a	0.00850	0.03577	0.11579	0.340	7.46	-0.38	7.84	0.20
10 b	0.00850	0.03577	0.11579	0.340	7.47	-0.36	7.83	0.21
10 c	0.00850	0.03577	0.11579	0.340	7.47	-0.37	7.84	0.20
10 d	0.01232	0.03190	0.10802	0.329	7.26	-0.57	7.83	0.21
11	0.01004	0.04227	0.13683	0.370	7.45	-0.38	7.83	0.21
11 a	0.01004	0.04227	0.13683	0.370	7.43	-0.39	7.82	0.22
11 b	0.01004	0.04227	0.13683	0.370	7.44	-0.37	7.81	0.23
11 c	0.01004	0.04227	0.13683	0.370	7.44	-0.37	7.81	0.23
12	0.01159	0.04877	0.15790	0.397	7.42	-0.38	7.80	0.24
12 a	0.01159	0.04877	0.15790	0.397	7.42	-0.39	7.81	0.23
12 b	0.01159	0.04877	0.15790	0.397	7.43	-0.35	7.78	0.26
12 c	0.01159	0.04877	0.15790	0.397	7.43	-0.37	7.80	0.24
12 d	0.01159	0.04877	0.15790	0.397	7.43	-0.37	7.80	0.24
12 e	0.01690	0.04346	0.14728	0.384	7.22	-0.58	7.80	0.24

in the electronic environment. Thus all salts are reduced to the same basis of number and charges of the ions. Where the ionic strength principle holds, individual differences in salt effects vanish.

If we follow out the curves for brom-cresol purple (Fig. 6) when the diluting salts are KCl and Na₂SO₄, all of the ions being different in kind, and the salts in valence type, it is apparent that the

TABLE XVIII.

Phenol Red pK' Change at 20° with Increase of Ionic Strength by Means of Sodium Chloride. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: phosphate as in Sample 3, Table XVII, + NaCl.	μ	$\sqrt{\mu}$	Electro-metric p_{H^+} .	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta \text{pK}'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.01575	0.125	7.61	-0.32	7.93	0.11
1 a	0.00000	0.01575	0.125	7.64	-0.33	7.97	0.07
1 b	0.00000	0.01575	0.125	7.59	-0.34	7.93	0.11
2	0.00905	0.02480	0.157	7.59	-0.33	7.92	0.12
2 a	0.00905	0.02480	0.157	7.57	-0.34	7.91	0.13
3	0.01810	0.03386	0.184	7.54	-0.34	7.88	0.16
3 a	0.01810	0.03386	0.184	7.56	-0.34	7.90	0.14
3 b	0.01810	0.03386	0.184	7.54	-0.35	7.89	0.15
4	0.02716	0.04292	0.207	7.51	-0.35	7.86	0.18
4 a	0.02716	0.04292	0.207	7.52	-0.36	7.88	0.16
5	0.04528	0.06106	0.247	7.47	-0.37	7.84	0.20
5 a	0.04528	0.06106	0.247	7.48	-0.38	7.86	0.18
6	0.06343	0.07922	0.281	7.43	-0.40	7.83	0.21
6 a	0.06343	0.07922	0.281	7.43	-0.40	7.83	0.21
7	0.08159	0.09738	0.312	7.40	-0.42	7.82	0.22
7 a	0.08159	0.09738	0.312	7.40	-0.41	7.81	0.23
8	0.10886	0.12466	0.353	7.36	-0.43	7.79	0.25
8 a	0.10886	0.12466	0.353	7.35	-0.43	7.78	0.26
9	0.13617	0.15197	0.390	7.32	-0.46	7.78	0.26
9 a	0.13617	0.15197	0.390	7.32	-0.45	7.77	0.27
10	0.16352	0.17934	0.423	7.29	-0.47	7.76	0.28
10 a	0.16352	0.17934	0.423	7.27	-0.48	7.75	0.29

variation in pK' mainly depends on the concentration in terms of $\sqrt{\mu}$. The two curves are very close together for the two different salts. However, if the molal unit of concentration is taken, a

TABLE XIX.

Phenol Red pK' Change at 20° with Increase of Ionic Strength by Means of Potassium Chloride. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: phosphate as in Sample 3, Table XVII, + KCl.	μ	$\sqrt{\mu}$	Electro-metric $p\alpha_{II}^{+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	μ per kg H_2O						
1	0.00000	0.01575	0.125	7.59	-0.36	7.95	0.09
1 a	0.00000	0.01575	0.125	7.60	-0.34	7.94	0.10
2	0.00905	0.02480	0.157	7.57	-0.34	7.91	0.13
3	0.01810	0.03386	0.184	7.54	-0.38	7.92	0.12
3 a	0.01810	0.03386	0.184	7.55	-0.36	7.91	0.13
4	0.02716	0.04292	0.207	7.52	-0.39	7.91	0.13
4 a	0.02716	0.04292	0.207	7.51	-0.37	7.88	0.16
5	0.04528	0.06106	0.247	7.48	-0.39	7.87	0.17
5 a	0.04528	0.06106	0.247	7.48	-0.39	7.87	0.17
6	0.06343	0.07922	0.281	7.44	-0.39	7.83	0.21
6 a	0.06343	0.07922	0.281	7.44	-0.40	7.84	0.20
7	0.08159	0.09738	0.312	7.41	-0.41	7.82	0.22
7 a	0.08159	0.09738	0.312	7.42	-0.42	7.84	0.20
8	0.10886	0.12466	0.353	7.39	-0.43	7.82	0.22
8 a	0.10886	0.12466	0.353	7.39	-0.43	7.82	0.22
9	0.13617	0.15197	0.390	7.33	-0.44	7.77	0.27
9 a	0.13617	0.15197	0.390	7.35	-0.44	7.79	0.25
10	0.16352	0.17934	0.423	7.31	-0.45	7.76	0.28
10 a	0.16352	0.17934	0.423	7.32	-0.44	7.76	0.28

great discrepancy arises. An apparent difference due to the kind of salt used is obtained almost in the very beginning of the curves at great dilutions.

It is clear that ionic strength is as closely correlated to variation in the activity coefficients of these indicators as it is to variations

TABLE XX.

Phenol Red pK' Change at 20° with Increase of Ionic Strength by Means of Sodium Sulfate. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: phosphate as in Sample 3 or 3b, Table XVII, + Na ₂ SO ₄ .	μ	$\sqrt{\mu}$	Electrometric p α H ⁺ .	Colorimetric color ratio: log R.	pK' indicator.	Δ pK'
	<i>M per kg. H₂O</i>						
1	0.00000	0.01579	0.126	7.62	-0.32	7.94	0.10
1 a	0.00000	0.01579	0.126	7.59	-0.33	7.92	0.12
1 b	0.00000	0.01579	0.126	7.62	-0.32	7.94	0.10
1 c	0.00000	0.01472	0.121	7.62	-0.53	7.95	0.09
2	0.00302	0.02485	0.158	7.60	-0.32	7.92	0.12
2 a	0.00302	0.02379	0.154	7.40	-0.54	7.94	0.10
3	0.00537	0.03190	0.179	7.56	-0.32	7.88	0.16
3 a	0.00604	0.03389	0.184	7.53	-0.35	7.88	0.16
3 b	0.00604	0.03389	0.184	7.56	-0.35	7.91	0.13
3 c	0.00604	0.03283	0.181	7.36	-0.35	7.91	0.13
4	0.00906	0.04295	0.207	7.54	-0.36	7.90	0.14
5	0.01343	0.05606	0.237	7.50	-0.35	7.85	0.19
5 a	0.01509	0.06106	0.247	7.50	-0.36	7.86	0.18
5 b	0.01509	0.05999	0.245	7.31	-0.56	7.87	0.17
6	0.01880	0.07219	0.269	7.45	-0.37	7.82	0.22
6 a	0.02113	0.07916	0.281	7.48	-0.37	7.85	0.19
7	0.02417	0.08830	0.297	7.44	-0.38	7.82	0.22
7 a	0.02717	0.09728	0.312	7.42	-0.38	7.80	0.24
7 b	0.02717	0.09728	0.312	7.44	-0.37	7.81	0.23
7 c	0.02717	0.09622	0.310	7.44	-0.57	7.81	0.23
8	0.03223	0.11247	0.335	7.42	-0.38	7.80	0.24
8 a	0.03622	0.12445	0.353	7.39	-0.39	7.78	0.26
8 b	0.03622	0.12445	0.353	7.42	-0.38	7.80	0.24
8 c	0.03622	0.12339	0.351	7.22	-0.58	7.80	0.24
9	0.04028	0.13663	0.369	7.38	-0.39	7.77	0.27
9 a	0.04528	0.15161	0.389	7.35	-0.40	7.75	0.29
9 b	0.04528	0.15161	0.389	7.38	-0.40	7.78	0.26
9 c	0.04528	0.15055	0.388	7.18	-0.60	7.78	0.26
10	0.04834	0.16079	0.401	7.36	-0.41	7.77	0.27
10 a	0.05433	0.17878	0.423	7.32	-0.42	7.74	0.30

TABLE XXI.

Phenol Red pK' Change at 20° with Increase of Ionic Strength by Means of Potassium Sulfate. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: phosphate as in Sample 3 or 3 b, Table XVII, + K ₂ SO ₄ .	μ	$\sqrt{\mu}$	Electro-metric $p\alpha_{H^+}$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>M per kg. H₂O</i>						
1	0.00000	0.01579	0.126	7.62	-0.34	7.96	0.08
1 a	0.00000	0.01579	0.126	7.59	-0.34	7.93	0.11
1 b	0.00000	0.01472	0.121	7.42	-0.53	7.95	0.09
2	0.00227	0.02259	0.150	7.60	-0.35	7.95	0.09
2 a	0.00227	0.02259	0.150	7.61	-0.34	7.95	0.09
2 b	0.00227	0.02259	0.150	7.57	-0.34	7.91	0.13
2 c	0.00227	0.02152	0.147	7.40	-0.54	7.94	0.10
3	0.00453	0.02938	0.172	7.57	-0.37	7.94	0.10
3 a	0.00453	0.02938	0.172	7.59	-0.35	7.94	0.10
3 b	0.00453	0.02938	0.172	7.55	-0.35	7.90	0.14
3 c	0.00453	0.02832	0.168	7.38	-0.55	7.93	0.11
4	0.00679	0.03618	0.190	7.55	-0.38	7.93	0.11
4 a	0.00679	0.03618	0.190	7.62	-0.27	7.89	0.15
4 b	0.00679	0.03618	0.190	7.54	-0.36	7.90	0.14
4 c	0.00755	0.03738	0.193	7.37	-0.56	7.93	0.11
5	0.01133	0.04976	0.223	7.52	-0.38	7.90	0.14
5 a	0.01133	0.04976	0.223	7.59	-0.28	7.87	0.17
5 b	0.01133	0.04976	0.223	7.52	-0.36	7.88	0.16
5 c	0.01133	0.04976	0.223	7.51	-0.36	7.87	0.17
5 d	0.01133	0.04870	0.221	7.33	-0.57	7.90	0.14
6	0.01586	0.06335	0.251	7.49	-0.39	7.88	0.16
6 a	0.01660	0.06559	0.253	7.56	-0.29	7.85	0.19
6 b	0.01660	0.06559	0.253	7.50	-0.36	7.86	0.18
6 c	0.01586	0.06335	0.251	7.50	-0.37	7.87	0.17
7	0.02039	0.07695	0.277	7.46	-0.40	7.86	0.18
7 a	0.02265	0.08374	0.286	7.44	-0.39	7.83	0.21
7 b	0.02265	0.08374	0.286	7.54	-0.30	7.84	0.20
7 c	0.02265	0.08374	0.286	7.48	-0.37	7.85	0.19
7 d	0.02039	0.07695	0.277	7.47	-0.37	7.84	0.20

TABLE XXI—*Concluded.*

Sample No.	Composition: phosphate as in Sample 3 or 3 b, Table XVII, + K_2SO_4 .	μ	$\sqrt{\mu}$	Electro-metric $\text{p}\alpha\text{H}^+$.	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta \text{pK}'$
	<i>m per kg. H₂O</i>						
8	0.03019	0.10636	0.323	7.41	-0.39	7.80	0.24
8 a	0.03019	0.10636	0.323	7.50	-0.31	7.81	0.23
8 b	0.03019	0.10636	0.323	7.45	-0.37	7.82	0.22
8 c	0.02718	0.09733	0.312	7.46	-0.37	7.83	0.21
9	0.03398	0.11771	0.343	7.39	-0.43	7.82	0.22
9 a	0.03776	0.12907	0.355	7.39	-0.40	7.79	0.25
9 b	0.03776	0.12907	0.355	7.47	-0.33	7.80	0.24
9 c	0.03398	0.11771	0.343	7.43	-0.38	7.81	0.23
10	0.04077	0.13810	0.371	7.36	-0.44	7.80	0.24
10 a	0.04682	0.15625	0.391	7.37	-0.41	7.78	0.26
10 b	0.04682	0.15625	0.391	7.44	-0.34	7.78	0.26
10 c	0.04077	0.13810	0.371	7.42	-0.38	7.80	0.24
11	0.05588	0.18343	0.423	7.35	-0.42	7.77	0.27
11 a	0.05588	0.18343	0.423	7.40	-0.37	7.77	0.27

in the activity coefficients of the salts of simple organic and inorganic acids. Whatever differences exist in the "salt errors" caused by different salts at equal ionic strengths may or may not be attributable to individual specific ion or molecule effects.

Individual Specific Ion Effect.—To what extent specific ion effects are present in these systems is not clear, nor may one decide the question from these experiments alone. In Fig. 2, for brom-cresol green, NaCl and KCl follow along practically the same curve as does the pair Na_2SO_4 and K_2SO_4 , and the pair MgCl_2 and CaCl_2 ; and this latter, in spite of the fact that MgCl_2 has an enormous effect, through hydrolysis, on the $\text{p}\alpha\text{H}^+$ of buffer solutions as compared with the other salts. The acetate mixture curve is of a slightly different shape. The curves for the various salts seem to show an indicator activity coefficient variation due to valence type differences in the salts of the surrounding medium. As the solution is more concentrated, the effects become more pronounced. The glucose experiment (Table VIII), indicates a

TABLE XXII.

Phenol Red pK' Change at 20° with Increase of Ionic Strength by Means of Magnesium Chloride. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: phosphate as in Sample 3, Table XVII, + MgCl.	μ	$\sqrt{\mu}$	Electro-metric $p\alpha_{H^+}$	Colorimetric color ratio: $\log R$.	pK' indicator.	$\Delta pK'$
	<i>μ per kg. H₂O</i>						
1	0.00000	0.01472	0.121	7.63	-0.31	7.94	0.10
1 a	0.00000	0.01472	0.121	7.61	-0.32	7.93	0.11
2	0.00327	0.02452	0.157	7.49	-0.43	7.92	0.12
2 a	0.00327	0.02452	0.157	7.47	-0.45	7.92	0.12
3	0.00653	0.03432	0.185	7.37	-0.54	7.91	0.13
3 a	0.00653	0.03432	0.185	7.36	-0.56	7.92	0.12
4	0.01089	0.04738	0.218	7.25	-0.64	7.89	0.15
4 a	0.01089	0.04738	0.218	7.23	-0.66	7.89	0.15
5	0.01633	0.06370	0.252	7.15	-0.71	7.86	0.18
5 a	0.01633	0.06370	0.252	7.14	-0.73	7.87	0.17
6	0.02286	0.08329	0.289	7.05	-0.77	7.82	0.22
6 a	0.02286	0.08329	0.289	7.03	-0.77	7.80	0.24
7	0.02939	0.10288	0.321	6.99	-0.82	7.81	0.23
7 a	0.02939	0.10288	0.321	6.97	-0.84	7.81	0.23
8	0.03918	0.13227	0.364	6.91	-0.88	7.79	0.25
8 a	0.03918	0.13227	0.364	6.89	-0.89	7.78	0.26
9	0.04898	0.16165	0.402	6.85	-0.92	7.77	0.27
9 a	0.04898	0.16165	0.402	6.83	-0.92	7.75	0.29
10	0.05877	0.19104	0.437	6.79	-0.96	7.75	0.29
10 a	0.05877	0.19104	0.437	6.77	-0.96	7.73	0.31

constant level of pK at constant salt concentration unaffected by variation in sugar concentration.

Fig. 3, for brom-cresol purple, is very much like that for brom-cresol green; the relationships, however, are a little different.

TABLE XXIII

Phenol Red pK' Change at 20° with Increase of Ionic Strength by Means of Magnesium Sulfate. Colorimetric Readings with Indicator Concentration = 0.00002 M.

Sample No.	Composition: phosphate as in Sample 3, Table XVII, + MgSO ₄ .	μ	$\sqrt{\mu}$	Electrometric p ^H +	Colorimetric color ratio: log R.	pK' indicator	Δ pK'
	<i>M per kg. H₂O</i>						
1	0.00000	0.01472	0.121	7.61	-0.32	7.93	0.11
1 a	0.00000	0.01472	0.121	7.60	-0.32	7.92	0.12
2	0.00226	0.02378	0.154	7.53	-0.41	7.94	0.10
2 a	0.00226	0.02378	0.154	7.52	-0.41	7.93	0.11
3	0.00453	0.03283	0.181	7.45	-0.48	7.93	0.11
3 a	0.00453	0.03283	0.181	7.46	-0.48	7.94	0.10
4	0.00754	0.04489	0.212	7.37	-0.55	7.92	0.12
4 a	0.00754	0.04489	0.212	7.36	-0.55	7.91	0.13
5	0.01131	0.05998	0.245	7.28	-0.61	7.89	0.15
6	0.01659	0.08110	0.285	7.21	-0.67	7.88	0.16
6 a	0.01659	0.08110	0.285	7.22	-0.68	7.90	0.14
7	0.02263	0.10524	0.324	7.13	-0.71	7.85	0.19
8	0.03017	0.13540	0.368	7.07	-0.76	7.83	0.21
8 a	0.03017	0.13540	0.368	7.07	-0.76	7.83	0.21
9	0.03771	0.16558	0.407	7.01	-0.81	7.82	0.22
9 a	0.03771	0.16558	0.407	7.01	-0.80	7.81	0.23
10	0.04676	0.20178	0.449	6.96	-0.84	7.80	0.24
10 a	0.04676	0.20178	0.449	6.96	-0.84	7.80	0.24
11	0.05582	0.23798	0.488	6.91	-0.88	7.79	0.25
11 a	0.05582	0.23798	0.488	6.90	-0.87	7.77	0.27

MgSO₄ shows a tendency (more pronounced in the case of phenol red), to lower the pK' curve. The glucose experiment (Table XVI) showed no effect of the non-electrolyte, at least, in these concentrations. More concentrated sugar solutions would probably

TABLE XXIV.
Value of pK' Indicator Extrapolated to $\mu = 0$.

Brom-cresol green.		Brom-cresol purple.		Phenol red.	
Salt.	pK	Salt.	pK	Salt.	pK
NaCl	4.90	NaCl	6.46	NaCl	8.05
KCl	4.92	KCl	6.47	KCl	8.05
Na ₂ SO ₄	4.92	Na ₂ SO ₄	6.47	Na ₂ SO ₄	8.05
K ₂ SO ₄	4.91	K ₂ SO ₄	6.46	K ₂ SO ₄	8.05
NaC ₂ H ₃ O ₂	4.93	PO ₄	6.47	PO ₄	8.02
MgCl ₂	4.94	MgCl ₂	6.45	MgCl ₂	8.04
CaCl ₂	4.95	MgSO ₄	6.45	MgSO ₄	8.00
Average.....	4.92		6.46		8.04

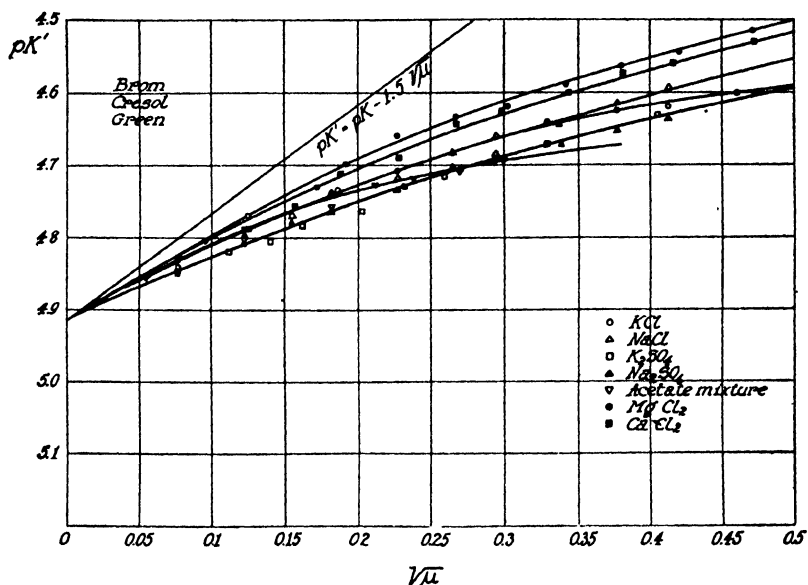


FIG. 2. The effect of varying concentrations of different salts on pK' of brom-cresol green.

affect the pK' as found by the methods used in this work, by causing a change in light absorption through the medium, and by altering the dielectric constant of the solution, the non-electrolyte exerting a salting-out effect.

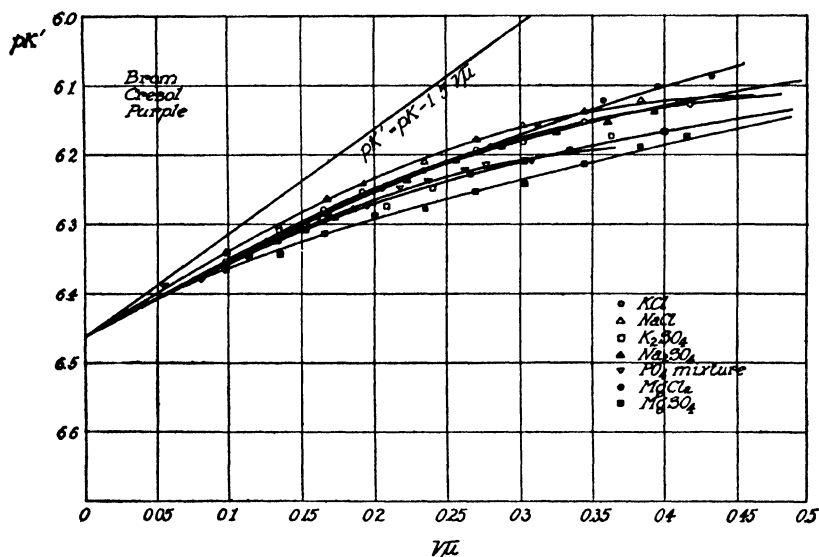


FIG 3 The effect of varying concentrations of different salts on pk' of brom-cresol purple

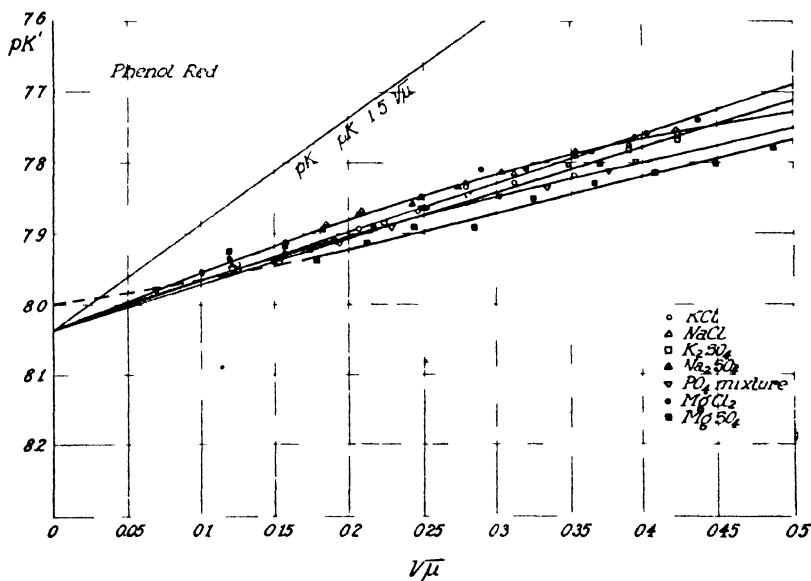


FIG 4 The effect of varying concentrations of different salts on pk' of phenol red

TABLE XXV.

Data of Lepper and Martin (26) Calculated to Show Phenol Red pK' Change at 18° with Increase of Ionic Strength.

Lepper and Martin table No.	Composition.		μ	$\sqrt{\mu}$	Electro-metric $p\alpha_{H^+}$.	Colori-metric $p\alpha_{H^+}$.	"Salt error" $p\alpha_{H^+} (c) - p\alpha_{H^+} (e)$.	$\Delta pK'$ indicator.
	Buffer.	Salt.						
I	Phosphate.							
	M/1000		0.00271	0.052	7.83	7.65	-0.18	7.96
	M/400		0.00680	0.082	7.81	7.66	-0.15	7.93
	M/150		0.01811	0.135	7.74	7.66	-0.08	7.86
	M/40		0.06791	0.260	7.65	7.61	-0.04	7.82
	M/15		0.18106	0.425	7.55	7.55	± 0.00	7.78
II	Phosphate.	NaCl						
	M/1000		0.01287	0.113	7.76	7.62	-0.14	7.92
	M/100		0.02143	0.148	7.74	7.61	-0.13	7.91
	M/32		0.04331	0.208	7.70	7.58	-0.12	7.90
	M/16		0.07576	0.275	7.61	7.56	-0.05	7.83
	M/8		0.13763	0.371	7.53	7.53	± 0.00	7.78
III	Phosphate.	NaCl						
	M ∞		0.02565	0.160	7.51	7.36	-0.15	7.93
	M/1000		0.02664	0.163	7.50	7.36	-0.14	7.92
	M/128		0.03350	0.183	7.45	7.33	-0.12	7.90
	M/32		0.05709	0.239	7.38	7.31	-0.07	7.85
IV	Bicarbonate.	NaCl						
	M/1000		0.02112	0.145	7.50	7.37	-0.13	7.91
	M/50		0.04022	0.201	7.48	7.38	-0.10	7.88
	M/16.6		0.08071	0.284	7.44	7.38	-0.06	7.84
	M/6.9		0.16601	0.407	7.42	7.41	-0.01	7.79
V	Bicarbonate.	NaCl						
			0.04030	0.201	7.51	7.41	-0.10	7.88
	M/30		0.07049	0.265	7.47	7.43	-0.04	7.82
	M/10		0.14091	0.375	7.44	7.43	-0.01	7.79

Phenol red curves (Fig. 4) present a slightly different picture. The average slope is somewhat lower than for the brominated methyl compounds, and all of the salts are much more closely grouped. $MgSO_4$, however, particularly at the lower concentrations, seems out of line. La Mer and coworkers (27) have found the same difficulty in working with salts of tri-trivalent type.

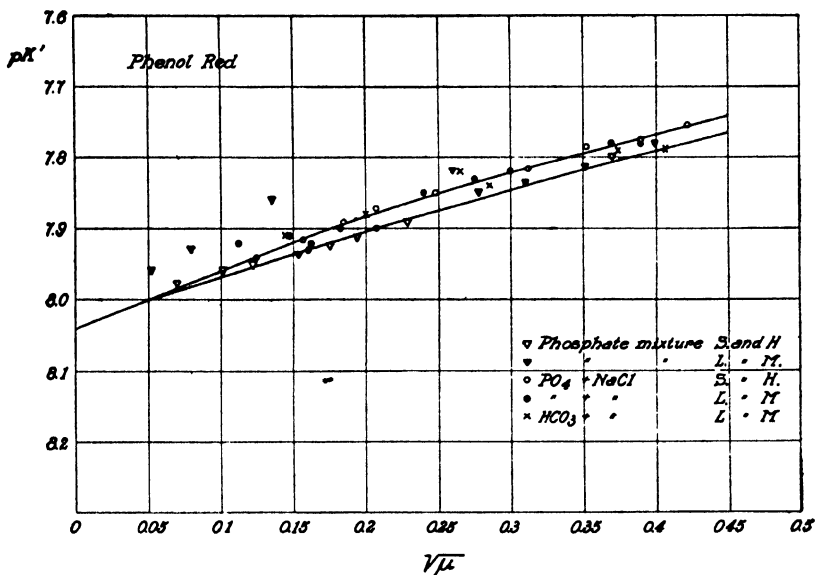


FIG. 5. Comparison of data of Lepper and Martin with those of present paper on change in pK' of phenol red.

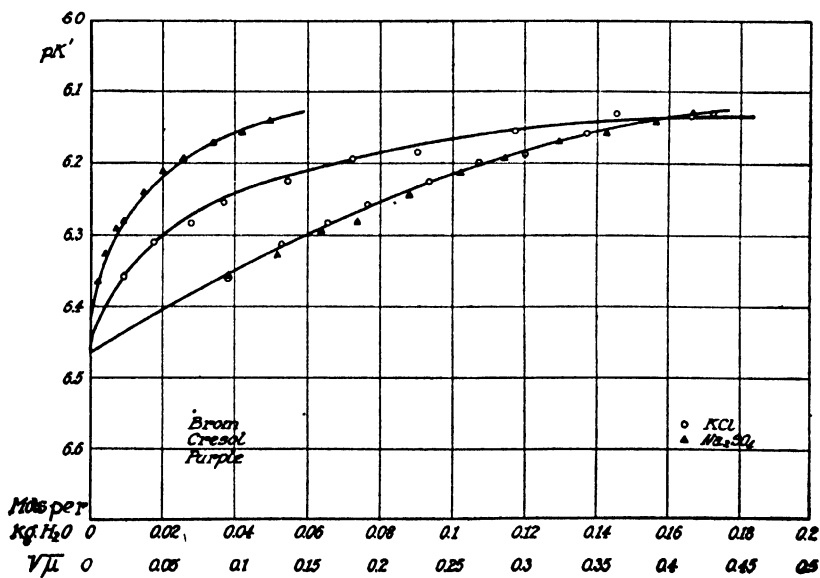


FIG. 6. The change in pK' of brom-cresol purple with KCl and Na_2SO_4 plotted against concentrations on molal basis and on ionic strength basis.

The foregoing experiments were performed with dilute solutions of acetate and phosphate mixtures. Although Lepper and Martin (26) make a distinction between results obtained with bicarbonate and phosphate buffers, their own data with NaCl (Fig. 5) in phenol red solutions show very good agreement between solutions of these two buffers. We did no experiments with bicarbonate buffer ourselves, but there is little reason to believe that the results would have been very different, provided the salt concentrations were calculated on the ionic strength basis. In physiological solutions the maximum concentration of about 30 mM bicarbonate would be $\sqrt{\mu} = 0.16$, where apparent individual salt or ion effects only begin to come into play. However, if further work indicates a real difference in effect of bicarbonate and phosphate, correction figures would have to take into account the nature of the buffer. Lepper and Martin's curve for phosphate effect is quite different from ours. Ours was established on the basis of 52 different points, and fits in well with the curves for other salts.

The whole series of experiments shows no very consistent specific ion or salt effects. For example, we find no common effect of Mg^{++} ion in MgCl_2 and MgSO_4 , or of SO_4^- ion in K_2SO_4 , Na_2SO_4 , and MgSO_4 .

Activity Coefficient Variation and Interionic Force Effects.—We have attempted to apply the equation of Debye and Hückel (Equation 8) to the curves of Figs. 2 to 4, but have not been completely successful in obtaining equations which would describe the curves throughout their entire course. The whole of the ionic dissociation and interionic attraction theory at the present time is in such a state of flux (29, 30) that we have attempted no modification such as would enable us to fit the data to the theory, or *vice versa*. Nernst (31) and his coworkers (32, 33), on the basis of the anomalous heats of dilution obtained with uni-univalent strong electrolytes, advocate a return to the old Arrhenius incomplete or partial dissociation theory, at the same time retaining the Debye-Hückel explanation for such effects as are due to interionic forces. Bjerrum (34) also has put forward a slightly different "association" explanation, and more recently (35) the idea that the effective dielectric decreases in the immediate neighborhood of the ions, thereby causing anomalous results. Gronwall, La Mer, and Sandved (36) have expanded the Debye-

Hückel formula in the form of an infinite series. They thereby eliminate negative ion sizes and obtain positive values even in the case of ions of small size. Simms (18), working with polyvalent weak electrolytes, has attempted to correct for deviations from the theory by taking into account a variable distance between the two charges of a di-anion.

In general, the curves obtained in Figs. 2 to 4 conform approximately to the equation of Debye and Hückel. Also, the correspondence between these curves and some of those obtained with weak acid buffer systems is very good. For example, the calculated curve of Cohn (16) based on the data of Sørensen, for the second dissociation constant of phosphoric acid, and the curve for the effect of NaCl on brom-cresol purple coincide almost exactly,

and have the equation $pK' = pK - \frac{1.5\sqrt{\mu}}{1 + 1.65\sqrt{\mu}}$

Also, there is here added proof of the dibasic character of these indicators. The equation for a monobasic indicator, with the assumption of a constant thickness of the ionic atmosphere = $0.33 \times 10^8 \sqrt{\mu}$ cm., would yield negative ion sizes. It is fairly certain that such a result is not due to the same factors which caused La Mer and his associates to expand the Debye-Hückel equation, for here the ion sizes are quite large.

Güntelberg and Schiödt (37) have recently published some work in which they state that the behavior of the activity coefficient of brom-phenol blue, the tetrabrom compound of the sulfon-phthalein series, is that of a monobasic acid. However, if we calculate from their single result at low concentrations, where $\frac{Kc}{Ka}$ corresponding to our $\frac{K'}{K}$ is given as 2.12 for a concentration of KCl = 0.1 N, $pK' = pK - 0.33$, which corresponds almost exactly to our results for brom-cresol purple and brom-cresol green. The treatment of these indicators as dibasic is consistent with their structure and known behavior.

Phenol red presents curves of a type somewhat different from those of brom-cresol purple and brom-cresol green. For this, we have no good explanation at the present time. Whether the difference is due to large ion size, or the absence of the methyl and bromide groups, we cannot say. Perhaps the results are due to the same reason which caused the evaluation of $1.6 \sqrt{\mu}$ instead of $2.0 \sqrt{\mu}$ for $-\log \gamma_{CO_3^{2-}}$ (15).

On the assumption of a *constant* value for the thickness of the ionic atmosphere, $0.33 \times 10^8 \sqrt{\mu}$ cm., we can make approximate calculations for ion size at $\sqrt{\mu} = 0.4$, this value changing with different types of salt present, with the ratio of alkaline to acid forms of the indicator, and with the concentration: from 5 to 8.7×10^{-8} cm. for brom-cresol green and brom-cresol purple, and from 8.7 to 13.0×10^{-8} cm. for phenol red. These values are merely speculative. Further developments in the theory of solutions, and particularly those concerned with buffers and weak electrolytes, may make it necessary to revise our treatment of these indicator anions, which, in structure at least, are so different from the simpler substances on which the modern theories are based. Furthermore, it must be remembered that not only ionization but tautomerism equilibria are here involved. The latter may not be affected to the same degree by electrostatic forces.

Activity Coefficient Variation and Indicator Color.—The recent work of Cohn and his collaborators on the phosphate and acetate buffer system activity coefficients indicates that the mol fraction partition of the two members of the system, *i.e.* the ratio $\frac{B_2A}{BA}$ or $\frac{BA}{HA}$, may also enter in as a factor governing the magnitude of the activity coefficient at any one given ionic strength. This variation would be in accord with Brönsted's specific ionic interaction theory. Applied to these indicator studies, this would mean that at any one given ionic strength, the pK' of the indicator would not be a constant, but would show some variation, depending on the color, *i.e.* the ratio of alkaline to acid form of the indicator, of the solution. Instead of *one* curve describing the variation of pK' of the indicator with changing ionic strength, one should obtain, for each salt studied (see Figs. 2 to 4), a family or narrow sheaf of curves. The width of such a sheaf of curves would be approximately the same for all salts, at the same ionic strength. Each one of these curves of any one family of curves would describe the pK' variation with ionic strength change, for the particular salt studied, *at a constant ratio of alkaline to acid form of the indicator; i.e.*, the curves would be isochromatic.

To obtain such isochromatic curves at varying values of $\log R$

(the logarithm of the ratio of alkaline to acid form of the indicator), the actual $p\alpha_{H^+}$ and the ionic strength of the particular salt solution used would progressively have to be so altered that the indicator color remained constant. In other words, the change in $p\alpha_{H^+}$ of the solution would have to be equal to, and of the same algebraic sign as the change in pK' of the indicator. Such a nice adjustment is impossible for these studies, since the two variables

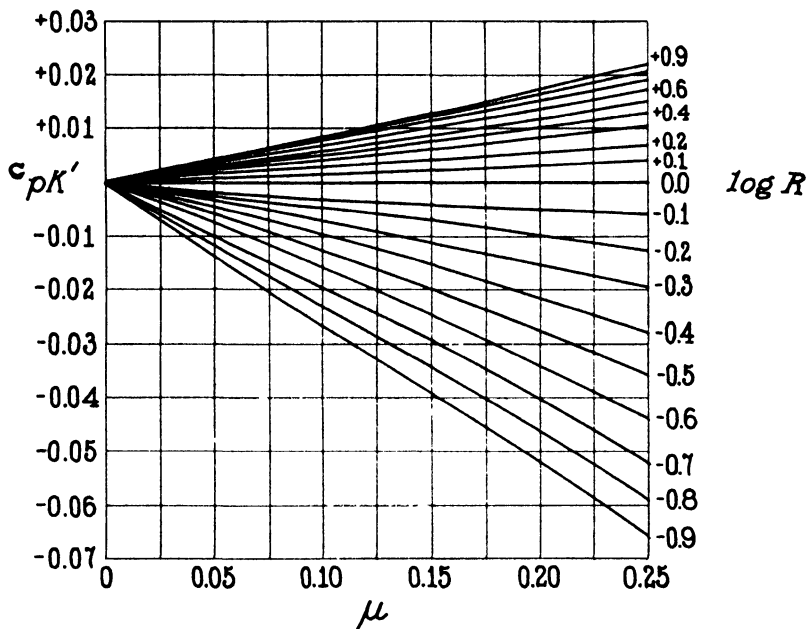


FIG. 7. Correction chart for values of pK' of the indicators at different ionic strengths, when the ratio (R) alkaline to acid form of the indicator varies. Adapted from Cohn's (16) data on phosphate buffer mixtures.

are not known, but are to be observed or calculated. Hence the curves given in Figs. 2 to 4 are not isochromatic nor do they cover the possible variation of pK' with ionic strength change between the two extremes of color within which the indicators may be used.

The indicator, in its acid and alkaline form, constitutes a buffer pair, and must so be treated. On the assumption that the $BA_2:BA$ or $BA:HA$ ratio of an indicator affects the pK' in the same way that the mol fraction partition of phosphate and acetate

buffers influences their respective pK' values, even when the indicator concentration is as low as in our experiments, one may correct for this factor in the following manner. Fig. 7 is interpolated from Cohn's data on the second dissociation constant of phosphoric acid, and applied to the indicators, which are also regarded here as dibasic. The figure shows the correction $c_{pK'}$, to be applied to the pK' value of the indicator at any ionic strength, as the logarithm of the ratio of the alkaline to acid form of the indicator varies. The experimentally determined pK' values at different ionic strengths, with different salts, have all been corrected to the value at $\log R = 0$, and are given in Tables XXVI to

TABLE XXVI.

Values of pK' of Brom-Cresol Green at 20° in Various Salts at Different Ionic Strengths, When $\log R = 0$. Extrapolated pK at $\mu = 0$ is 4.92.

μ	NaCl	KCl	Na ₂ SO ₄	K ₂ SO ₄	CaCl ₂	MgCl ₂	Acetate mixture.
0.025	4.77	4.77	4.78	4.79	4.75	4.73	4.78
0.050	4.72	4.71	4.74	4.74	4.69	4.67	4.73
0.075	4.68	4.67	4.70	4.70	4.65	4.63	4.71
0.100	4.65	4.65	4.68	4.68	4.62	4.60	4.69
0.125	4.63	4.63	4.66	4.66	4.59	4.58	4.67
0.150	4.61	4.62	4.64	4.64	4.57	4.56	
0.175	4.59	4.61	4.62	4.63	4.56	4.55	
0.200	4.57	4.59	4.60	4.61	4.53	4.52	
0.225	4.56	4.59	4.59	4.60	4.52	4.50	
0.250	4.55	4.58	4.57	4.59	4.51	4.49	

XXVIII. These are the pK' values to which $c_{pK'}$ is to be added when the indicator color is such that $\log R$ is different from zero. At the physiological ionic strength, $\mu = 0.16$, the corrections for the indicator pK values given in these tables, would vary throughout the useful range of the indicators, in the following manner: for brom-cresol green, from +0.01 to -0.03, for brom-cresol purple, from +0.01 to -0.02, for phenol red, from +0.01 to -0.04, depending on the value of $\log R$. At lower ionic strengths, the corrections would, of course, be less. It is apparent from Fig. 7 that this factor of the mol fraction partition is of much less importance when the predominating color is that of the alkaline form of the indicator.

The impossibility of systematically testing this effect for indicators has already been mentioned. However, the probable validity of such an effect for these substances also is indicated by the table given by Hastings and Sendroy (24) in which such consistency of pK' for phenol red in $m/15$ phosphate solutions

TABLE XXVII.

Values of pK' of Brom-Cresol Purple at 20° in Various Salts at Different Ionic Strengths, When $\log R = 0$. Extrapolated pK at $\mu = 0$ is 6.46.

μ	NaCl	KCl	Na ₂ SO ₄	K ₂ SO ₄	MgCl ₂	MgSO ₄	Phosphate mixture.
0.025	6.28	6.29	6.30	6.31	6.30	6.32	6.30
0.050	6.22	6.23	6.24	6.26	6.23	6.28	6.25
0.075	6.18	6.19	6.20	6.23	6.19	6.25	6.22
0.100	6.15	6.17	6.18	6.21	6.16	6.23	6.20
0.125	6.14	6.15	6.16	6.19	6.14	6.21	6.19
0.150	6.13	6.14	6.14	6.17	6.12	6.19	6.18
0.175	6.12	6.13	6.13	6.16	6.11	6.18	
0.200	6.11	6.12	6.12	6.15	6.11	6.17	

TABLE XXVIII

Values of pK' of Phenol Red at 20° in Various Salts at Different Ionic Strengths, When $\log R = 0$. Extrapolated pK at $\mu = 0$ is 8.04.

μ	NaCl	KCl	Na ₂ SO ₄	K ₂ SO ₄	MgCl ₂	MgSO ₄	Phosphate mixture.
0.025	7.91	7.93	7.92	7.93	7.93	7.94	7.93
0.050	7.86	7.87	7.87	7.88	7.89	7.91	7.90
0.075	7.84	7.86	7.85	7.85	7.86	7.89	7.87
0.100	7.82	7.84	7.82	7.83	7.84	7.88	7.86
0.125	7.80	7.81	7.80	7.81	7.82	7.87	7.84
0.150	7.79	7.79	7.78	7.79	7.81	7.86	7.81
0.175	7.78	7.78	7.77	7.78	7.79	7.85	7.80
0.200	7.78	7.77	7.76	7.78	7.77	7.84	7.79
0.225		7.76		7.77	7.77	7.84	

through the range of $p\alpha_{H^+}$ from 6.84 to 7.98 was obtained. From Table XXVIII one would expect the increase in μ from 0.1200 to 0.1740 in their experiments, at constant color, to cause a progressive decrease in pK' indicator of about 0.04 unit. However, the $p\alpha_{H^+}$ of the $m/15$ phosphates varied with the ionic strength, and the consequent color change was such that $\log R$ varied from -0.94 at $\mu = 0.120$ to $+0.20$ at $\mu = 0.174$. Fig. 7 indicates that between these limits the pK' will progressively increase by the same

amount; namely, 0.04 unit. The resultant of the two effects would be the constant pK' for phenol red obtained throughout the whole range.

Results Applied to Colorimetric Estimation of $p\alpha_{H^+}$ of Salt Solutions.—It is apparent that these indicators possess definite physical characteristics which are much like those of other weak acids or alkali buffer systems with their salts, and are affected in approximately the same way by changes in the medium by which they are surrounded. Hence it is no longer necessary to refer to the "salt error," when all that is meant is that the activity coefficients of the colored anions, for the same general class of salts, are different at different ionic strengths, and at different proportions of the alkaline to acid form of the indicator.

In salt solutions of known ionic strength, one should be able to estimate the pK' of these indicators to within ± 0.02 unit, and to determine $p\alpha_{H^+}$ with nearly the same accuracy. The "salt error," $p\alpha_{H^+}(c) - p\alpha_{H^+}(e)$, has been a source of discrepancy, owing to failure to allow for a predictable effect of interionic forces on the apparent dissociation constants of the indicators in standard and unknown solutions.

Practically, one may calculate, *when using bicolor standards at 20° ,*

$$(9) \quad \begin{array}{ccc} p\alpha_{H^+} = pK' \text{ of indicator} & + & c_{pK'} \text{ at } \log R \text{ of indicator} + \log R \\ \text{of sample} & \text{of sample} & \text{at } \mu \text{ of sample} \end{array}$$

where $\log R$ is the logarithm of the ratio of the alkaline to acid form of the indicator, and $c_{pK'}$ is the correction in pK' when $\log R$ is not zero. The values pK' , in salt solutions of varying μ , when $\log R = 0$, are found in Tables XXVI to XXVIII. The values of $c_{pK'}$ when $\log R \neq 0$ are found by referring to Fig. 7.

If instead of bicolor standards, standards with buffer and indicator in the same solution are used, the assumed or electrometrically determined $p\alpha_{H^+}$ value of the standard buffer-indicator solution also enters in:

$$(10) \quad \begin{array}{ccccccc} p\alpha_{H^+} \text{ of} & = & p\alpha_{H^+} \text{ of} & + & pK' \text{ of indicator} & + & c_{pK'} \text{ at } \log R \text{ of} \\ \text{sample} & & \text{standard} & & \text{at } \mu \text{ of sample} & & \text{indicator at } \mu \text{ of} \\ & & & & & & \text{sample} \\ & & & & - & pK' \text{ of indicator} & - & c_{pK'} \text{ at } \log R \text{ of indicator} \\ & & & & & \text{at } \mu \text{ of standard} & & \text{at } \mu \text{ of standard} \end{array}$$

For most purposes, the $c_{pK'}$ corrections of Equation 10 may be neglected. Nevertheless, it is obvious that colorimetric readings of salt solutions may be simplified and made more accurate by using bicolor standards made up and marked in even color ratio ($\log R$) series, and calculating $p\alpha_{H^+}$ according to Equation 9. Table XXIX is given to facilitate the preparation of such a series of standards for any indicator.

Although these indicator dissociation constants have been obtained at 20° , the results may also be applied to determinations at 38° or at any intermediate temperature. In previous work (24, 25), brom-cresol green was found to have practically no temperature coefficient for pK' . Brom-cresol purple changed -0.10 unit and phenol red -0.13 unit in pK' when the temperature was increased from 20° to 38° . These temperature coefficients should be the same throughout the ionic strength range from zero to $\mu = 0.2$.

On the basis of these results, we may examine some previous results in the field of colorimetric $p\alpha_{H^+}$ determinations. The pK' for phenol red in $M/15$ phosphate solutions at 20° was found by Hastings and Sendroy to be 7.78. For the same phosphate solutions we obtain here a revised value of 7.80. This correction is probably due to the actual change in $p\alpha_{H^+}$ of the phosphates (about $+0.02$ to $+0.01$) when diluted 10 per cent, a refinement in technique not used in previous determinations. In like manner, the pK' at 20° for brom-cresol purple previously found for the same phosphate solutions would be changed from 6.19 to the value 6.21 found in the present work.

The acetates are not affected by dilution to the extent that phosphates are. In fact, the results of the present determinations show pK' of brom-cresol green at 20° to be 4.67 for acetate solution instead of 4.72 for the ionic strength of 0.140. What this discrepancy may be due to is not known. This indicator, as was previously noted, is not always of constant purity.

In the development of a $p\alpha_{H^+}$ method for body fluids (24) we previously noted that the ionic strength of the *bicolor standards* (aside from the effect of salt on optical absorption) was of no moment. This we can now better understand. If the acidity and alkalinity of the two tubes are sufficient to keep the color of the indicator in total acid and total alkaline form respectively,

TABLE XXIX.

Rounded Values of Log $\frac{\text{Alkaline Form}}{\text{Acid Form}}$ at Intervals of 0.05 or 0.1 $\text{p}\alpha\text{H}^+$ Unit for Use in Preparing Bicolor Standards.

Alkali tube.		Acid tube.		Log R.
Indicator.	Alkali.	Indicator.	Acid.	
cc.	cc.	cc.	cc.	
0.18	24.82	2.32	22.68	-1.10
.20	.80	.30	.70	-1.05
.23	.77	.27	.73	-1.00
.25	.75	.25	.75	-0.95
.28	.72	.22	.78	-0.90
.31	.69	.19	.81	-0.85
.34	.66	.16	.84	-0.80
.38	.62	.12	.88	-0.75
.42	.58	.08	.92	-0.70
.46	.54	.04	.96	-0.65
.50	.50	2.00	23.00	-0.60
.55	.45	1.95	.05	-0.55
.60	.40	.90	.10	-0.50
.66	.34	.84	.16	-0.45
.71	.29	.79	.21	-0.40
.77	.23	.73	.27	-0.35
.83	.17	.67	.33	-0.30
.90	.10	.60	.40	-0.25
.97	.03	.53	.47	-0.20
1.04	23.96	.46	.54	-0.15
.11	.89	.39	.61	-0.10
.18	.82	.32	.68	-0.05
.25	.75	.25	.75	0.00
.32	.68	.18	.82	+0.05
.39	.61	.11	.89	+0.10
.46	.54	.04	.96	+0.15
.53	.47	0.97	24.03	+0.20
.67	.33	.83	.17	+0.30
.79	.21	.71	.29	+0.40
.90	.10	.60	.40	+0.50
2.00	23.00	.50	.50	+0.60
.08	22.92	.42	.58	+0.70
.16	.84	.34	.66	+0.80
.22	.78	.28	.72	+0.90
.27	.73	.23	.77	+1.00
.32	.68	.18	.82	+1.10

regardless of how the indicator dissociation curve may be shifted by some change in ionic strength or temperature, the bicolor standards will always have the same color ratio. The conclusion from our previous results on addition of salt to bicolor standards, namely that the pK' of phenol red would be 0.02 unit *lower* at zero ionic strength than in $M/15$ phosphate solution, is, of course, erroneous.

The difference of -0.05 between colorimetric determinations of dilute bicarbonate solutions at 20° and 38° , given in the same paper (24), was to be expected. The carbonic acid pK' , and hence the actual $p\alpha_{H^+}$ would be decreased 0.18 unit at the higher temperature, while the indicator pK' would decrease only 0.13 unit. The color reading would therefore be 0.05 unit lower.

CONCLUSIONS.

The data given in this paper represent, we believe, an advance in the systematic tabulation of the experimental effect of salts on the colors of three indicators widely used in biological work. The practical application of these findings holds regardless of the degree of validity of the theory involved.²

Until there is an extension, to other types of indicators, of this method of studying the effect of salt concentration on indicator color, it would be premature to advocate the application of these theories to every indicator. All that may be stated at the present time is that there are three indicators of the sulfonphthalein series, which, on the basis of extensive experimental work, seem to behave as do other typical weak electrolytes. The results are well in accord with recent studies involving electrolyte behavior, and also with Ostwald's dissociation theory of indicator color.

² As this paper is about to go to press, we find that Kolthoff reports (*J. Physic. Chem.*, **32**, 1820, December (1928)) the result of a study of the "salt error" of several indicators, from which he concludes that the salt correction of indicators cannot be adequately calculated from the equation of Debye and Hückel. That the ionic strength of solutions alone is inadequate to account for the activity coefficients of ions in general is well recognized. It seems from his results as well as those presented in this paper that the treatment of "salt errors" as a problem of activity coefficients of weak electrolytes provides the best explanation for their existence and magnitude.

We are indebted to Mr. Gilbert Stone for his careful determination of the electrometric $p\alpha_{H^+}$ values in this paper.

SUMMARY.

1. By means of electrometric $p\alpha_{H^+}$ estimations, and color readings against bicolor standards, the effect of NaCl, KCl, Na_2SO_4 , K_2SO_4 , $MgCl_2$, $CaCl_2$, $MgSO_4$, acetate and phosphate buffer mixtures, and glucose, on the activity coefficients of the sulfonphthalein indicators, brom-cresol green, brom-cresol purple, and phenol red, has been studied.

2. With the indicators, as with other weak acids in the presence of their salts, the value of pK' is dependent upon the total electrolyte content of the solution. It is because of this fact that addition of neutral salts alters the color of an indicator solution.

3. When the effects of salts on indicators are correlated with ionic strengths, salts of different valence types show at the same ionic strengths approximately like effects on the pK' of the indicator.

4. Quantitatively the relation between ionic strength and pK' is expressed by the equation of Debye and Hückel, based on the assumption that all the salts are completely dissociated and that the combined effect of their ions on the log of an activity coefficient (in this case on the change they produce in pK') is proportional, as a first approximation, to the square root of the ionic strength.

5. Our results indicate that other, lesser, factors than ionic strength influence the relationship between $p\alpha_{H^+}$ and indicator color. Among such factors may be varying specific interionic effects of individual ions or groups of ions. Such effects are, however, in most cases, relatively slight compared with the main, non-specific effect indicated by the ionic strength.

6. Tables and equations are given for the determination of $p\alpha_{H^+}$ colorimetrically, which take into account possible variations in the salt content either of the sample or of the standards, or of both. A correction is also applied for the ratio in which the alkaline and acid forms of the indicator are present in solution.

7. The results are applied to a discussion of various phases of recent work in colorimetric $p\alpha_{H^+}$ estimations.

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GROWTH OF RATS ON "FAT-FREE" DIETS.

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In a series of studies on *The Relation of Diet to the Quality of Fat Produced in the Animal Body*, it was desirable to ascertain if possible, the composition of the fat yielded by an animal grown under a dietary régime in which there was no ingested fat. Obviously tissue fat formed under such conditions represents a product synthesized in the organism from precursors other than dietary fat. This product might be designated as "physiological" fat in contrast with exogenous fat transferred more or less unchanged from the food to the tissues. Accordingly, in order that such "synthetic" fat might be available for analysis, albino rats were grown on a ration practically devoid of fat. Under the conditions outlined, the growing of animals presented certain outstanding difficulties—notably, the necessity of feeding the so called fat-soluble vitamins without their commonly accompanying fat vehicles. The present communication refers to the methods employed and the degree of success attained in surmounting the difficulties encountered.

The Experiments.

Experimental Animals.—With the exception of three females, as indicated on the charts, male albino rats taken at weaning, when approximately 21 days of age and weighing from 35 to 45 gm., were selected from our laboratory stock animals.¹ With these rats—which were kept individually, some in cylindrical

* Holder of the Alpha Xi Delta Fellowship awarded by the American Association of University Women (1928-29).

¹ The laboratory stock animals were grown at that time on the Sherman Diet B, modified (Smith, A. H., and Bing, F. C., *J. Nutr.*, 1, 179 (1928)).

false bottom wire cages and others in cages, consisting largely of glass, specially designed to facilitate cleaning—extensive precautions were taken to minimize contamination by fatty materials. In some cases as indicated on the charts, food was withheld during the 2 days immediately after weaning, before giving the experimental diet, in order that a partial depletion of the stored fat might ensue, thereby lessening its influence on the later resulting "synthetic" fat. In order to exclude, as much as possible, variations due to individual litters, the rats of a given litter were fed different dietary supplements.

All animals were weighed twice weekly.

Experimental Diets.—The selected diets, consisting of a "basal" diet, fed *ad libitum*, and "accessories," quantitatively supplied daily apart from the "basal" food, had the following composition:

Partially Purified.

1. 18 per cent casein, commercial.
2. 77 " " sucrose.
3. 5 " " salt mixture.

100 per cent by weight.

"Fat-Free" (and Modifications).

"Basal" Diet.

1. 18 per cent casein, purified.
2. 77 " " sucrose.
3. 5 " " salt mixture.

100 per cent by weight.

"Accessories."

- | | |
|--|---|
| <ol style="list-style-type: none"> 4. Liver extract (\approx 0.4 gm. of dried liver). 5. Yeast 200 mg. 6. Cod liver oil concentrate 20 mg. | <ol style="list-style-type: none"> 4. Liver extract (\approx 0.4 gm. of dried liver). 5. Yeast concentrate 100 mg. 6. Cod liver oil concentrate 20 mg., or one of the following replacements: <ol style="list-style-type: none"> (a) Cod liver oil concentrate 20 mg. + peanut oil 20 mg. (b) Cod liver oil 20 mg. (c) " " " 20 " + peanut oil 20 mg. 7. Irradiated ergosterol 0.016 mg. |
|--|---|

1. *Casein* was chosen as the protein because after it is subjected to a process of purification, involving solution, reprecipitation, and washing with alcohol and ether a number of times, the fat content may be reduced below 0.2 per cent as determined by the modified Roese-Gottlieb method (Shaw, 1920). The commercial casein gave a value of 1.7 per cent of fatty materials. Inasmuch as good, though not optimal growth has resulted when

casein is incorporated in certain diets to the extent of 18 per cent (Osborne and Mendel, 1926), it seemed best for the purposes of this experiment to feed the casein at a level no higher than 18 per cent, in order to keep the possible contaminants at a minimum.

2. *Sucrose* replaced starch, the usual source of carbohydrate in rat diets, because starch contains not only associated fat but also combined fat which can be extracted with ether only after hydrolysis (Taylor and Nelson, 1920). That rats would eat a diet consisting of a high percentage of sugar was indicated in a study by Evans and Burr (1926-27). The highest grade of granulated sugar, ground to a powder, was incorporated in the diets.

3. The *salt mixture* used was that of Osborne and Mendel (1919).

4. A hot water *extract of pig liver*,² concentrated in a partial vacuum at a low temperature, was given in view of the excellent growth promoted through the use of this dietary supplement, by Osborne and Mendel (personal communication). In the partially purified diet, this extract was administered incorporated with starch in small pills, while in the "fat-free" diet the extract, preserved with alcohol, was fed as such. The amount of fatty materials in a daily dose of this extract was approximately 1.5 mg.³

5. *Harris yeast concentrate*, because of its lower fat content (less than 0.4 per cent of ether-soluble substances as determined by a Soxhlet extraction), replaced in the "fat-free" diet the yeast used in the partially purified diet. This lot of concentrate fed at the level of 70 mg. has been shown in this laboratory to promote the growth of rats to 120 gm. in 120 days, when it is fed as a source of vitamin B to animals depleted of their store of this dietary essential. Realizing that failure in growth might be due to the inadequacy of the yeast preparation in the so called vitamin B complex, after doubling in two cases the dose of the concentrate, we supplemented it later with 400 mg. of yeast daily.

6. The *cod liver oil concentrate*,⁴ a non-saponifiable fraction of

² A large part of the extract used was kindly supplied by Dr. A. J. Wakeman of the Connecticut Agricultural Experiment Station.

³ This analysis was carried out by extracting in a Soxhlet apparatus with ether, strips of paper (Carl Schleicher and Schüll No. 571) on which was absorbed and dried a measured quantity of the liver extract.

⁴ We wish to thank Dr. H. E. Dubin of the H. A. Metz Laboratories for this concentrate, oscodal, a semisolid material from which the market

that oil, kept in an amber bottle and in an atmosphere of carbon dioxide, was weighed out daily to minimize oxidation. An examination of this material showed that 90 per cent of it could be recovered in the form of unsaponifiable matter.⁵ 20 mg. of the concentrate represent approximately the vitamin potency of 2 cc. of cod liver oil ("New and Nonofficial Remedies," 1927, 121).

7. To supply additional vitamin D, *irradiated ergosterol*⁶ was fed in preference to irradiating the animals directly, because of the great time factor involved in the daily irradiation of a large number of animals. This preparation was administered dissolved in absolute alcohol saturated with carbon dioxide, and the solution, made up weekly, was kept under carbon dioxide. In determining the potency of this sample of ergosterol irradiated dry, a positive line test resulted when the material was fed dissolved in olive oil in the amount of 0.001 mg. for 5 days to rats made rachitic on the Steenbock Diet 2965.⁷ Because, in the testing of this preparation, the irradiated ergosterol was fed dissolved in oil, it seemed advisable, in the absence of a natural fat vehicle, to feed daily a larger quantity than the test indicated potent.

A careful record of the food consumed was made, the "basal" diet being moistened to the consistency of a paste, to prevent spilling. For the duration of the greater part of the experimental period the "basal" food was weighed out daily—the food cups being filled with a gm. or two more than the individual rats had eaten the day previously, as determined by weighing the unconsumed food. The "accessories," mixed all together on a small dish, were administered daily before the remainder of the food, and were usually consumed immediately—thereby lessening the possibility of deterioration of the easily oxidizable supplements.

product Oscodal Tablets is made; also for a supply of cod liver oil of the same lot used in the preparation of the non-saponifiable fraction.

⁵ This figure was obtained by using the modified Kerr-Sorber method (*J. Oil and Fat Ind.*, **3**, 64 (1926)). After removal of the unsaponifiable material, the soap solution, upon acidification and extraction with ether, yielded 6 per cent of ether-soluble material, deeply pigmented.

⁶ This supply of tested irradiated ergosterol was kindly furnished by Dr. C. N. Frey of the Fleischmann Laboratories.

⁷ For the composition of the Steenbock Diet 2965 see Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 274 (1925).

It is estimated from the analyses of the dietary components that those rats receiving the "fat-free" diet with the cod liver oil concentrate, received daily approximately 7 mg. of fatty materials (exclusive of the 90 per cent non-saponifiable matter from the cod liver oil concentrate).

Distilled water was supplied *ad libitum* to all rats.

Plan of Experiments.—The forty-seven animals used in this study were divided into three groups—the first of which (Rats 1 to 7 inclusive) served merely as an orientation experiment. No attempt was made to secure extreme refinement of the ration fed them because this preliminary experiment was designed primarily to find out whether a cod liver oil concentrate could be utilized as a source of vitamin A when it was fed with a diet of low fat content. Interest was added to this question when Munsell and Black (1928) reported that certain cod liver oil preparations contain negligible amounts of vitamin A. Attempts were made at intervals (as indicated in Chart 1) to accelerate growth by doubling various components of the diet—yeast, liver extract, and finally the percentage of casein.

The second group of animals (Rats 8 to 21) was fed the "fat-free" diet with the cod liver oil concentrate. After about the 130th day of age, these rats received irradiated ergosterol.

The third series of animals (Rats 22 to 47 inclusive) was selected to answer three questions.

1. Would the growth of animals fed a supplement of cod liver oil concentrate be exceeded in rate of gain by those rats receiving the concentrate administered with a small amount of fat such as a vitamin A-free peanut oil? Hart, Steenbock, and coworkers (1926-27), and Daniels and Brooks (1926-27), have indicated that possibly the non-saponifiable fraction of cod liver oil is better utilized by an organism when the preparation is fed dissolved in an oil, than when it is administered without a fat vehicle.

2. Would better growth ensue in the rats fed cod liver oil of the same lot used in the preparation of the concentrate, than in those animals receiving the concentrate alone—both supplements fed at the same level, *i.e.* 20 mg.? The answer might give some indication of the relative potency of the two vitamin A supplements or of the deficiency of the diet.

3. Would the growth curves of animals fed a supplement of cod

liver oil compare favorably with those of animals receiving the cod liver oil plus a small amount of vitamin A-free peanut oil? On the assumption that the peanut oil contains at most negligible quantities of vitamin A, any difference noted here may be said to be due to the effect of the inclusion of a small amount of fat *per se* or to the possible presence of other vitamins in the peanut oil.

At designated intervals, the rats were killed by illuminating gas and a necropsy performed with observations on the eyes, tongue, bones, stomach, lungs, liver, bladder, and kidneys.

Experimental Results.

I. That the particular cod liver oil concentrate used in this study, when fed at a level of 20 mg. daily, served as an effective source of vitamin A is indicated by the growth curves. Chart 1 which represents graphically the gains made by those animals fed the partially purified diet, shows that four rats (Nos. 2 to 5), receiving the cod liver oil concentrate, attained "fairly" normal weights, while the control (Rat 7), deprived of a supplementary source of vitamin A, died, subsequent to the development of characteristic deficiency symptoms (xerophthalmia and loss in weight). One rat (No. 6) depleted of its store of vitamin A and then fed 100 mg. of the concentrate daily during 7 days, followed thereafter by 20 mg. daily, showed a clearing up of the eye disorder and a resumption in growth. Rat 1 which received 20 mg. of the concentrate daily during the first 7 days of the experiment only, has a curve of "average" growth. This record might be taken as an argument for the storage of the concentrate, inasmuch as the other sources of vitamin A available, namely the impurities of the diet and reserve store from the mother, did not suffice for continued growth in Rat 7 which received the same diet, without the initial administration of the concentrate.

Of the animals on the purified ration, two control animals of Group II, deprived of a source of vitamin A died, showing the characteristic symptoms of vitamin A deficiency; whereas two other rats, depleted of their store of vitamin A and then receiving the concentrate, made good recovery (Chart 2). Four animals (Rats 28, 45, 24, 44) also resumed their growth when the cod liver oil preparation was administered after vitamin A depletion. Rat 27, receiving daily 100 mg. of the non-saponifiable fraction of cod

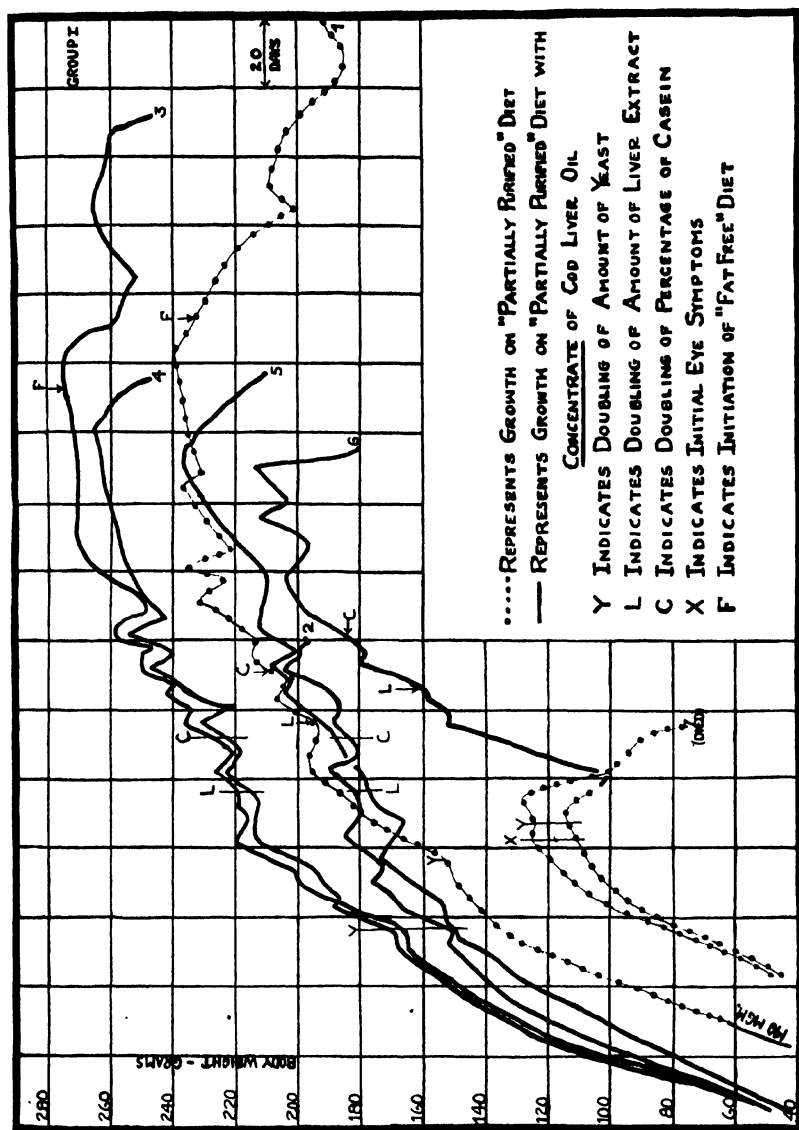


CHART 1.

liver oil during the 2 days in the beginning of the experiment, and Rat 43, 20 mg. daily during the first 7 days of the experimental period, both showed delayed onset of the symptoms of deficiency due to vitamin A, as compared with their litter mates, Rats 28 and 45 respectively, similarly grown with the exception of the initial feeding of a small amount of the cod liver oil concentrate (Chart 3).

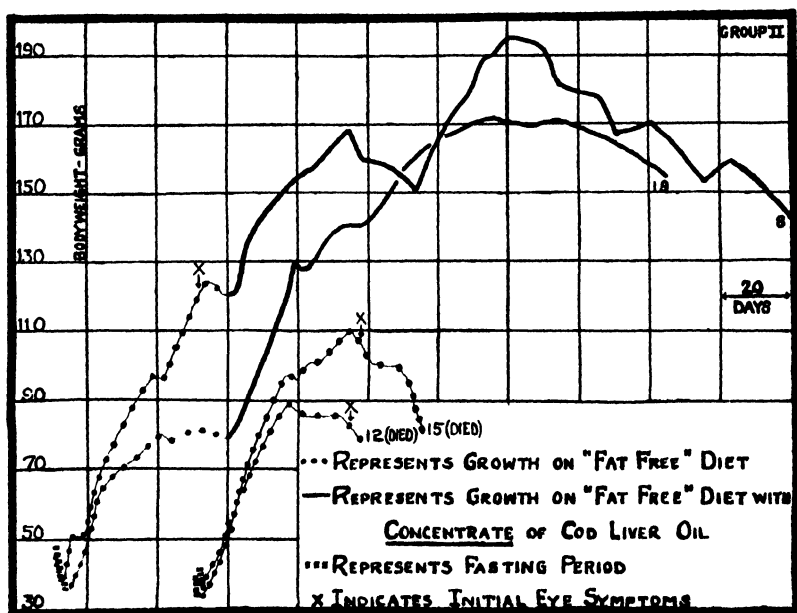


CHART 2.

From these data, it seems reasonable to assume that the non-saponifiable fraction of cod liver oil, fed at a level of 20 mg. daily, was an effective source of vitamin A. The possibility that the effectiveness of the concentrate is due to some contamination by the occlusion of a small amount of cod liver oil, is not entirely ruled out. If it is assumed that the 10 per cent of the cod liver oil concentrate not recovered in the unsaponifiable fraction (in our determination of the unsaponifiable matter in the concentrate) was all cod liver oil, this would mean that a rat might possibly

receive 2 mg. of cod liver oil in the daily ingestion of 20 mg. of concentrate. If the growth of the rats were limited by the cod liver oil supplement alone, then one should expect much better growth in those animals receiving 20 mg. of cod liver oil—10 times the amount possible in the case of those rats fed the concentrate. From Charts 4 to 6, it is seen that there is only a very little difference in the growth curves of animals fed supplements of cod liver oil and the concentrate of that oil respectively.

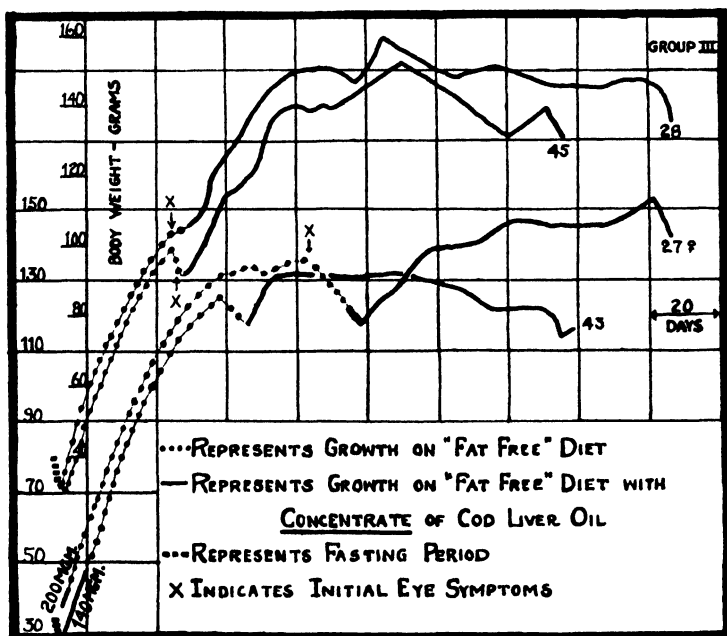


CHART 3.

II. The effect of an addition of a small amount of fat is illustrated from a study of the growth curves in Charts 5 to 8. From a comparison of Charts 5 and 6, it is seen that the animals receiving cod liver oil demonstrated growth a little better than those fed the same weight of the concentrate made from this lot of cod liver oil. Slightly better growth was made by those rats receiving a drop (about 20 mg.) of peanut oil in addition to the concentrate (Chart 7) than those given the concentrate alone (Chart 5)

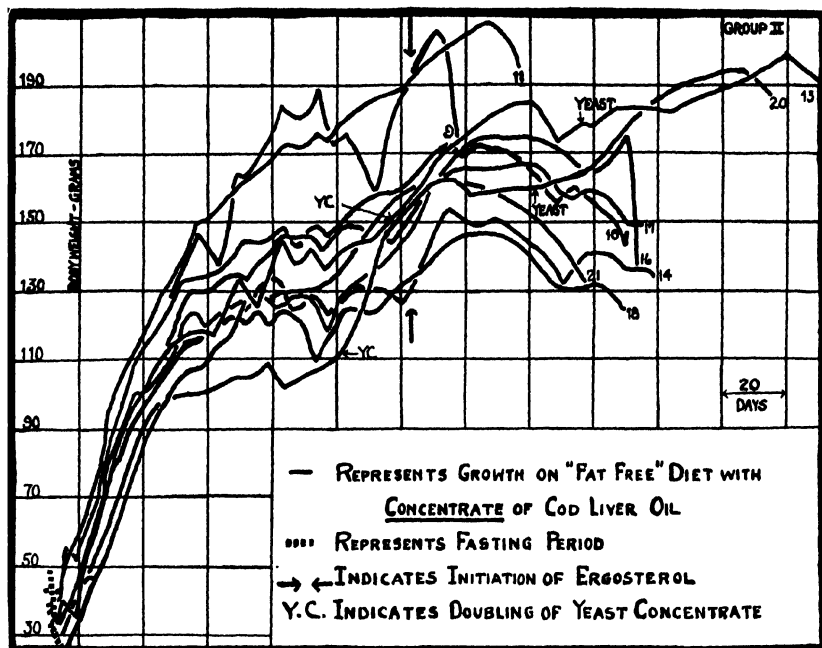


CHART 4.

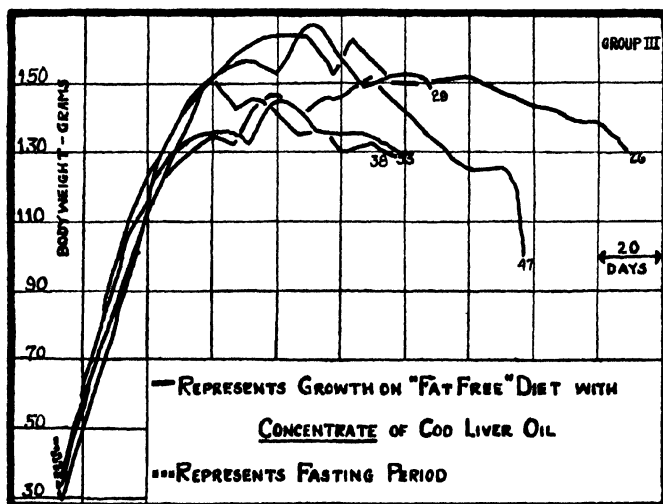


CHART 5.

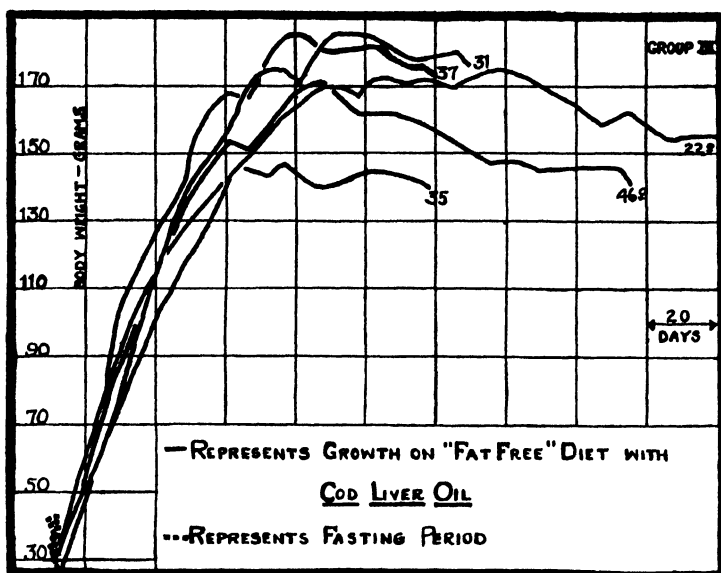


CHART 6.

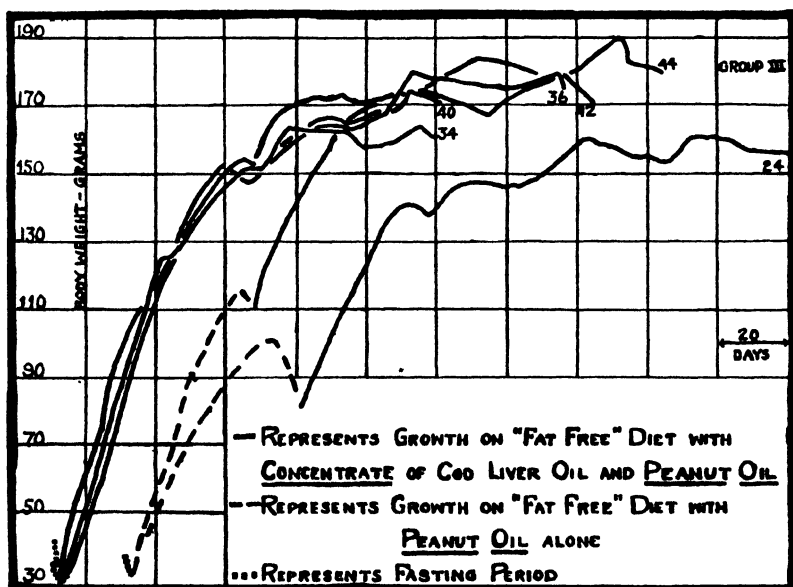


CHART 7.

and comparable growth was made by the former rats and those animals receiving only the cod liver oil. However, the best growth curves obtained represent the gains made by those rats whose diet was supplemented by 1 drop each of cod liver oil and peanut oil. Whether the beneficial effect of the addition of peanut oil is due to a small amount of vitamins (known or un-

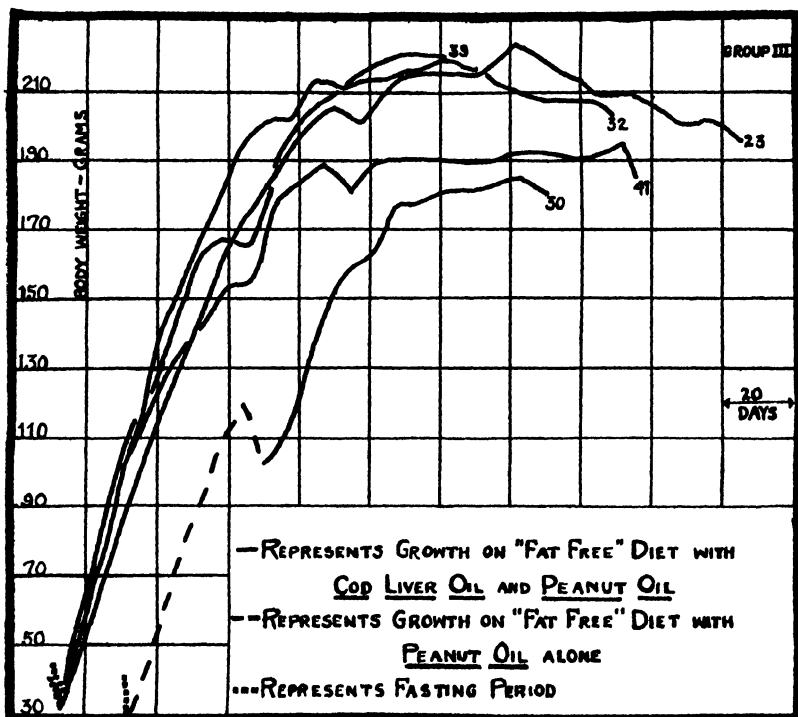


CHART 8

known) contained in it; to the aid fat may render in the absorption of the fat-soluble vitamins; or to the presence of the fat *per se*, is undetermined. The vitamin A content of the peanut oil was not enough to prevent decline in weight of the three rats administered 1 drop of this peanut oil as a sole source of vitamin A, and the animals did resume growth when the oil was supplemented with a vitamin A-bearing material (see Charts 7 and 8). Peanut oil incorporated in the diet to a very much larger extent—22 per

cent—seems to indicate that this oil does not contain any appreciable amount of vitamin A (Holmes, 1923).

A comparison of the growth curves of animals fed supplements of cod liver oil concentrate, alone, and with peanut oil, and cod liver oil both with and without peanut oil, indicates that those animals receiving the most fat grew best.

III. A few outstanding findings were noted in the necropsies:

1. A gelatinous mass was found in the bladders of one-half of the animals grown with the cod liver oil concentrate as compared with only one out of five of the animals receiving supplements of either cod liver oil or cod liver oil plus peanut oil. Some of these masses were very soft, while others were rather firm and resembled stones.

The kidneys of half the animals on the "fat-free" diet plus the cod liver oil concentrate were mottled, and in some cases, there were indentations in the surface of the kidney. Urinary calculi were observed in three rats (Nos. 6, 8, 19) first depleted of their store of vitamin A and then fed cod liver oil concentrate. The stones were found, in two of the animals, in the pelvis of the kidney, and in the third rat, several large stones were in the bladder and one small stone in the right ureter. The right kidney of this animal was only a shell, after the removal of the green pus which filled it (Fig. 1). These findings suggest that all damage due to dietary deficiencies is not completely repaired upon supplying the lacking component of the food— an illustration of a fact not always appreciated in the quantitative tests of the vitamin content of foods, as all "curative" methods may be rendered ineffective by previous irremedial conditions due to the depletion in an organism of a dietary essential.

2. Abnormality in the appearance of the lungs was observed in more than half of the rats receiving from the beginning of the experimental period the cod liver oil concentrate without additional fat, whereas none of the other animals administered a source of vitamin A with fat showed gross changes in this organ.

3. Hair balls were present in the stomachs of eleven rats out of the forty-seven observed. Mitchell, Bradshaw, and Carlson (1924) state that "formation of hair balls is attributed to high fat content and sticky consistency of the food." Our observations can at most confirm only the latter portion of their state-

ment—namely, that referring to the sticky consistency of the food.

On the living animals, grown on the "fat-free" diet with the cod liver oil concentrate, certain observations were made—such

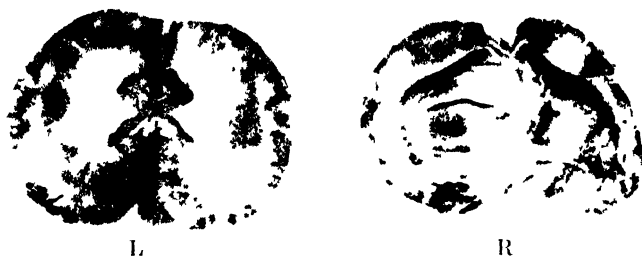


FIG. 1 Kidneys from rat (No. 6) grown on the partially purified diet and fed cod liver oil concentrate, *only after depletion of the body's store of vitamin A*



FIG. 2. Eye conditions observed. *A*, Rat 28, "control," grown on "fat-free" diet—just before administration of the cod liver oil concentrate. Age 59 days; weight 106 gm. *B*, Rat 14, grown on the "fat-free" diet with cod liver oil concentrate. Age 132 days; weight 125 gm. Note the rim about the edge of the eyelids. *C*, Rat 0, "normal," grown on a diet in which 40 per cent of the calories was furnished by dried skimmed milk and 60 per cent by soy bean oil. This diet was supplemented by 400 mg of yeast and 1 drop of cod liver oil. Age 58 days; weight 125 gm.

as poor condition of the fur, and in some cases priapism and bloody urine. In one rat, about $\frac{1}{2}$ inch of the end of the tail was quite dark and appeared gangrenous. A peculiar condition about the eyes resulted in three of the rats fed the purified ration containing the non-saponifiable fraction of cod liver oil. The eyelids were pink and in general the eyes resembled those in the incipient

stages of xerophthalmia, but at no time was a picture of true xerophthalmia presented (Fig. 2).

Inasmuch as most of the abnormalities noted occurred in those animals receiving the least fat, the argument for the beneficial effect of fat is strengthened.

IV. The fact that, regardless of the supplements used, optimal growth did not result in the animals reared on a diet extremely poor in fats, might be attributed to the inadequacy of one or more components of the diet. The inclusion of a source of vitamin E might have promoted growth (Evans, 1928). Likewise the feeding of casein at a higher level might have made possible an increase in body weight. The limit of the ability of the rat to utilize as high a concentration of sucrose as this diet contains may have been overstepped, thereby rendering less efficient some of the ingested food. Those animals in Group III fed the cod liver oil preparation do not show better growth than the rats in Group II in spite of the fact that in the former group irradiated ergosterol was administered throughout the experimental period, whereas the other rats received it only after about the 130th day (see Charts 4 and 5). Possibly irradiated ergosterol may not be utilized when it is fed in a non-fat medium as in a diet devoid of fat. The animals, however, did not appear to be rachitic, as was to be expected inasmuch as a good salt mixture was used. Irradiation of the animals directly probably would have been more satisfactory but even then the ability of an organism to respond to the irradiation to which it is subjected might be lessened when the animal is grown on a diet extremely poor in fatty materials. The inadequacy of the source of the vitamin B complex might account for the growth of the animals on the purified diet being inferior to that of the rats fed the partially purified diet. However, the addition of 400 mg. of yeast, from the same lot fed to the rats which were receiving the partially purified diet, did not produce marked increase in growth (Chart 4, Rats 13 and 20). Unfortunately, it is not known what type of growth curve would have resulted had the yeast been fed as a source of vitamin B components from the beginning.

SUMMARY AND CONCLUSION.

In order to study the composition of body fat yielded by an animal grown under a dietary régime in which there was no in-

gested fat, a diet of extremely low fat content (consisting of extracted casein, sucrose, Osborne and Mendel salt mixture, yeast concentrate, cod liver oil concentrate, irradiated ergosterol, and hot water extract of liver), has been fed to albino rats. Comparatively good, though by no means optimal growth has been recorded for these animals. The best growth, however, was exemplified by those animals which received some small inclusion of fat in their diet. Whether this apparent beneficial effect of a small amount of fat is due to its content of vitamin A or other vitamins, or to its action as a vehicle for the fat-soluble vitamins, or whether fat *per se* is essential, is not conclusively demonstrated.

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THE REACTION OF THE BLOOD IN CANCER.*

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Review of Experimental Methods.

The data recorded and opinions expressed regarding the relation between the reaction of normal blood and blood obtained from cancer patients are somewhat conflicting.

The methods generally employed may be classified as electro-metric methods including the use of the hydrogen electrode, quinhydrone electrode, and more recently the glass electrode, and the colorimetric method including those of Cullen and Dale and Evans (see Austin and Cullen (1)).

The hydrogen electrode when employed with correctly equilibrated serum appears to be fairly satisfactory as Bayliss, Keridge, and Verney (2) and more recently Earle and Cullen (3) and Cullen and Earle (4) have found. The first named workers state, however, that in many instances the hydrogen electrode may give fallacious results.

Again, the quinhydrone electrode, on the whole, appears to be satisfactory when used in serum. For example Cullen and Biilmann (5), while unable to obtain satisfactory results for whole blood with the quinhydrone electrode, contrary to the findings of Corran and Lewis (6), nevertheless, found that the quinhydrone electrode behaved quite as satisfactorily as the hydrogen electrode with human serum. At a later date, Earle and Cullen (3) and Cullen and Earle (4) questioned this finding, but more recently Mecker and Reinhold (7) presented evidence supporting the original

* Communicated by Professor W. C. M. Lewis. This investigation was undertaken on behalf of the Liverpool Medical Research Organization: Director, Professor W. Blair Bell, of the University of Liverpool.

contention of Cullen and Büllmann. It would appear, therefore, that the evidence is in favor of the use of the quinhydrone electrode for determining the reaction of serum, which is confirmed by Bayliss, Kerridge, and Verney (2) already referred to, and suggests that data obtained with whole blood with the quinhydrone electrode may be of some value when the readings are obtained almost immediately on mixing the blood and quinhydrone, as was done by Corran and Lewis. In this connection it should be noted that Mislowitzer (8) was able to employ the quinhydrone electrode with slightly diluted whole blood. In the case of whole blood, of course, the chief difficulty encountered is that of the drifting potential which has been fully investigated by Kuang-Liu (9).

The glass electrode first successfully used by Mrs. Kerridge (10) for animal tissues has been found by Bayliss, Kerridge, and Verney (2) to be equally suitable for serum. The writer has used this method for human blood in the present investigation.

Turning now to colorimetric methods, we find that a considerable body of opinion is against the employment of these methods on account of the variability of the necessary corrections to be applied before the results can be reduced to values comparable with those obtained by electrometric methods. In this connection the work of Bennett (11) and Johnston (12) should be consulted.

Review of Data on Reaction of Blood of Normal Persons and of Those with Cancer.

We have already noted that very conflicting data are to be found in the literature on the relation of the reaction of the blood of normal persons and individuals with cancer. Chambers and Kleinschmidt (13) find a slightly increased alkalinity with carcinoma cases, their pH values being calculated from CO₂ content and CO₂ absorption curves. Using the Cullen colorimetric method, Myers, Schmitz, and Booher (14) found no difference in the reaction of blood obtained from normal persons and cancer patients, respectively, while Reding (15) on the other hand, also using Cullen's colorimetric method, observed an increased alkalinity in the case of blood from cancer patients. Menten (16), using a hydrogen electrode, also observed an increased alkalinity with

TABLE I.
pH Values for Normal Blood (Different Human Subjects).

7.29	7.36	7.35	7.36	7.35	7.40	7.31	7.37	7.29	7.33
7.34	7.35	7.35	7.33	7.33	7.28	7.32	7.33	7.30	7.33
Mean.									7.34

TABLE II.
pH Value of Blood from Cancer Patients.

Case No.	Sex.	pH	Diagnosis and previous treatment.
1	M.	7.43	Carcinoma of rectum;* colotomy before blood was withdrawn.
2	F.	7.27	Carcinoma of cervix.*
3	"	7.39	Recurrent carcinoma of breast.*
4	"	7.34	Myeloma of jaw;* clinical recurrence elsewhere.
5	"	7.35	Ovarian carcinoma.* Had lead injected 2 years previously.
6	M.	7.33	Carcinoma of rectum.†
7	"	7.35	" " " †
8	"	7.33	" " " †
9	"	7.39	" " bladder.†
10	F.	7.33	Recurrent carcinoma of breast.*
11	"	7.35	Carcinoma of breast with secondary of spine.
12	M.	7.35	Carcinoma of stomach; secondary nodules on peritoneum.†
13	F.	7.26	Carcinoma of thyroid.
14	M.	7.34	" " cecum.*
15	"	7.36	" " head of pancreas.†
16	F.	7.33	" " cervix.*
17	"	7.36	Ovarian carcinoma.†
18	"	7.32	Recurrent carcinoma of rectum.*
19	M.	7.31	(Sarcoma of stomach?). Secondary throughout both lungs (x-ray). Lead (0.05 gm.) injected 2 days previously.
20	F.	7.38	Carcinoma of breast.* Local excision of large old standing tumor 28 days previously.
Mean.....		7.34	

The cases marked (*) have been confirmed histologically (these, ten in number, give a mean pH value of 7.34); those marked (†) were diagnosed at operation; the remaining cases have been diagnosed clinically as some of them are inoperable.

blood from cancer cases, but it is questionable whether this work is very reliable on account of the unsatisfactory treatment of the blood prior to its being used with the hydrogen electrode. Using a quinhydrone electrode with whole blood, Corran and Lewis (6) reported no difference in pH between normal blood and that from cancer patients.

As it is difficult to assess the conflicting data already cited, the present investigation has been undertaken with a view to determining by means of the glass electrode, what difference, if any, is to be observed in the reaction of blood from normal and cancer-afflicted individuals. The blood has been withdrawn and retained under liquid paraffin in order to prevent loss of carbon dioxide. The pH measurements have been made at 18° on whole blood, by means of the glass electrode, within 15 minutes of withdrawal.

The method of using the glass electrode and the theory underlying it have already been given by the writer (17) in a paper dealing with pH determinations of normal and cancerous tissue.

In Table I pII values are given for normal whole blood of a number of male individuals, while in Table II pH values are recorded for whole blood derived from a number of cancer patients of both sexes. In most cases the quantity of blood used was about 0.5 cc. The blood was rapidly obtained by venous puncture from the forearm.

CONCLUSIONS.

The majority of the cancer cases indicated in Table II are fairly advanced, and it will be seen on comparing Tables I and II that there is no difference in the average pH of blood obtained from normal and cancerous individuals. Furthermore, it should be noted that we have been concerned with *untreated* cases.

Corran and Lewis, who obtained similar results, also studied untreated cases separately from treated cases, in spite of Reding's general criticism that previous workers had not investigated untreated cases of cancer.

It is suggested that discordant results obtained by other investigators are due mainly to less satisfactory experimental methods.

SUMMARY.

Experimental methods for determining the pH of blood are reviewed, together with the application of these methods by other workers to determine the reaction of blood from human, normal and cancer subjects.

In the present investigation, data obtained by means of the glass electrode are presented to show that no difference in pH is to be observed between normal blood and blood from cancer patients.

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PENTOSE METABOLISM.

III. A COMPARISON OF THE RATES OF DISPOSAL OF *d*-ARABINOSE AND *l*-ARABINOSE IN THE RABBIT.*

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It has seemed of interest to follow the observations previously made with *l*-arabinose (Corley, 1928) with others made with its optical isomer. Neuberg and Wohlgemuth (1902) have expressed the opinion that *l*-arabinose resembles glucose more closely than does the *d* form. In support of this belief they reported the findings that following administration by various routes, the amounts of *d*-arabinose escaping utilization exceeded those for *l*-arabinose. While there is no direct evidence in this connection, and indeed such interconversions *in vitro* may bear no relationship to those carried out in the body, it would seem not unlikely that the aldehyde (free or potential), presumably the most labile group, would be the point of attack for lengthening or shortening the chain of carbon atoms. On this basis *d*-arabinose is the more similar to glucose. The view-point of Neuberg and Wohlgemuth has seemed questionable and their results surprising. Since *d*-xylose has appeared to enter the blood stream in considerable quantities after enteral administration, while *l*-arabinose has not, the possibility has arisen that the effect with these difficultly metabolizable sugars might in some manner be related to the form.

A comparison of the fate in the body of *d*- and *l*-arabinose might then be of interest in regard to the question as to whether, in such sugars as these, differences in physiological properties might be

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attributable to variable degrees of similarity to the readily utilizable sugars with 6 carbon atoms. The present communication records a study of the *d*-arabinose concentrations in the blood and urine following administration enterally and intravenously. The effect of insulin on the disposal of this sugar when injected intravascularly has been followed. Further observations have been made with *l*-arabinose, by using different dosages than previously and also by repeating certain of the experiments with another preparation of the compound.

TABLE I.
Effect of Intravenously Injected l-Arabinose on Blood Sugar.

Rabbit No.	50		68		67	
Weight, <i>kg.</i>	1.80		1.35		1.37	
Sugar administered, <i>gm.</i> ...	1.00*		2.00		2.74	
Time of fasting, <i>hrs.</i>	42		24		40	
	Total.	Unfermentable.	Total.	Unfermentable.	Total.	Unfermentable.
Blood sugar, <i>mg. per 100 cc.</i>						
Control period.	89	27	84	26	88	27
After sugar administration.						
3 min.	303	231	457	406	603	416
1 hr.	183	89	190	121	244	177
2 hrs.	129	52	130	55	142	74
3 "	126	36	130	36	121	41
Urine sugar, <i>mg.</i>	400	405	851	833	1282	1300

* Digestive Ferments Company preparation.

There have been employed the methods previously used for *l*-arabinose, changes in the amounts of unfermentable reducing substances being taken as an index of the amounts of the sugar present. The *d*-arabinose was obtained from the Eastman Kodak Company, and *l*-arabinose was prepared from mesquite gum, or was a Digestive Ferments Company preparation. $[\alpha]_D^{27}$ for *l*-arabinose was $+102^\circ$; $[\alpha]_D^{22}$ for *d*-arabinose was -104° . *d*-Arabinose manifested about the same resistance to fermentation by yeast as the *l* form, as it was possible to recover 95 to 105 per cent of the sugar added to blood or urine.

Results.

Intravenously injected *l*-arabinose has practically disappeared from the blood in 3 hours (Table I). As the dosage has been increased from 1 to 2 gm. per kilo of body weight, the amount remaining in the circulation has been somewhat greater at the end of this interval. In eight experiments, during this period of time from 40 to 50 per cent of the sugar has been recovered in the urine. The portion excreted has in general become larger with the higher dosages, although this difference has not been marked. Employ-

TABLE II.
Effect of Intravenously Injected d-Arabinose on Blood Sugar.

Rabbit No.....	55		62		68		55	
Weight, kg.....	1.65		1.30		1.32		1.50	
Sugar administered, gm...	1.00		2.00		2.64		1.00*	
Time of fasting, hrs.....	44		38		40		42	
	Total.	Unfermentable.	Total.	Unfermentable.	Total.	Unfermentable.	Total.	Unfermentable.
Blood sugar, mg. per 100 cc.								
Control period.	126	33	96	26	69	22	118	33
After sugar administration.								
3 min.	327	221	410	314	485	442	266	213
1 hr.	183	89	152	89	198	153	118	82
2 hrs.	139	47	118	39	123	74	84	44
3 "	142	39	99	24	116	35	99	30
Urine sugar, mg.	204	204	858	876	767	780	252	258

* With 2 units of insulin.

ment of a different preparation has yielded results in agreement as far as both blood picture and urinary excretion are concerned (Rabbit 50, Table I).

The removal from the blood of intravenously injected *d*-arabinose takes place at about the same rate as was observed for its optical isomer (Table II). While the disappearance of the former has seemed a little more rapid, the figures warrant no definite conclusions in this regard. However it is noteworthy that in the 3 hour experimental period, in nine cases but 20 to 44 per cent of

the amount injected has appeared in the urine, and furthermore that in only two instances have the amounts exceeded 32 per cent, namely 38 and 44 per cent, and these with the higher dosages. The simultaneous injection of insulin did not noticeably affect either the rate of removal of circulating *d*-arabinose or the amount recovered in the urine (Rabbit 55, Table II).

Following the enteral administration of *d*-arabinose, the total sugar of the blood was but slightly increased and the unfermentable

TABLE III.
Effect of Enterally Administered d-Arabinose on Blood Sugar.

Rabbit No.....	4		52	
Weight, <i>kg.</i>	1 32		1 50	
Sugar administered, <i>gm.</i> ...	1 32		3 00	
Time of fasting, <i>hrs.</i>	48		48	
	Total.	Unfermentable.	Total.	Unfermentable.
Blood sugar, <i>mg. per 100 cc.</i>				
Control period.	105	27	107	30
After sugar administration.				
1 hr.	110	33	123	35
2 hrs.	123	30	131	41
3 "	118	29	129	41
4 "	134	27	136	30
Urine sugar, <i>mg.</i>	94	94	180	179

fraction was practically unaffected with 1 gm. per kilo and but slightly increased after twice this amount (Table III).

It may be stated that the results obtained indicate that *d*-arabinose and *l*-arabinose are handled with about equal facility in the tissues of the rabbit, but that if there is any difference in ability of utilization, the *d* form seems the more readily attacked.

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THE OXIDATIVE DETERMINATION OF PHOSPHOLIPID (LECITHIN AND CEPHALIN) IN BLOOD AND TISSUES.

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With the increasing interest in the lipids as important factors in the essential processes of living matter, the need for accurate methods of measurement is too obvious to need comment. Especially needful are methods which can be used on small amounts of material such as can be obtained without great inconvenience from the living animal. Micro methods for phospholipids are already available for blood and these could be applied without difficulty to tissues as well. They are, however, based on determinations of the phosphoric acid content either by colorimetric or nephelometric measurements and are open to objection on the following grounds. (a) Phosphoric acid is only about one-eighth of the typical lecithin or cephalin molecule and it was early pointed out by Bang that it is more logical to use the remaining seven-eighths of the molecule in determining phospholipid. He was the first to use the oxidative method in putting this idea into practice (1). (b) It is assumed that all significantly important phospholipids are of the nature of lecithin and cephalin—monoamino monophosphatids—, while it is known that other types of phospholipid are found in tissues. (c) It is generally assumed that all alcohol- or ether-soluble phosphorus is of the lecithin-cephalin type of combination, while it has been pointed out (2) that other forms of phosphoric acid may be present to a considerable extent in such extracts. It may be stated that, while according to our present knowledge these assumptions are generally near enough to the truth for most practical purposes, the few analyses of tissues given in Table II indicate that they may sometimes be quite unsafe. A

method was sought which would involve neither of these assumptions, which would follow Bang's principle of measurement by the non-phosphate part of the molecule, and would make use as far as possible of standard processes (on a micro scale) for the isolation and determination. The oxidative procedure with chromic acid, first used by Bang and recently extensively modified (3), offered a satisfactory method of measurement, and the ordinary method of isolation of the phospholipids by precipitation from ether or petroleum ether with acetone and magnesium chloride has been found to give satisfactory results on mg. amounts of lecithin and cephalin.

Standard Substances.—Lecithin and cephalin were prepared from various sources as follows: Fresh tissue was extracted with hot alcohol in a continuous extractor, the alcohol evaporated under reduced pressure, the mixed lipids dissolved in a small amount of ether, the phospholipids precipitated by excess of acetone, and the precipitate collected by centrifuge. The precipitate was washed by kneading with acetone in the centrifuge tube to remove adherent fat and cholesterol, then dissolved in dry ether, and the separation and washing with acetone repeated twice more. The final precipitate of mixed lecithin and cephalin was dissolved in the minimum amount of dry ether and the cephalin precipitated with excess of absolute alcohol. The separated cephalin was washed by kneading with absolute alcohol, then dissolved in ether, and the precipitation with absolute alcohol repeated. The lecithin, which remained in solution in the alcohol-ether, was recovered by evaporation of the solvent in a vacuum at low temperature, dissolved in dry ether, and the alcohol precipitation repeated in smaller volume to remove traces of cephalin.

Each sample was then purified according to MacLean's method (4) by suspension in several volumes of water and precipitation with acetone together with a little NaCl. The flocculent precipitate was collected, dehydrated by kneading with acetone, dissolved in ether, precipitated with acetone, and then washed with acetone, after which the material was dissolved in dry ether and the precipitation repeated. The purified samples were dissolved in dry ether and made up to volume. For weight determination a suitable aliquot containing 100 to 200 mg. was measured into a dry, weighed flask, the solvent evaporated, the residue dried in a

stream of dry carbon dioxide, and the drying finished to constant weight in a vacuum desiccator. For the micro measurements a dilute standard solution of the substance was made by measuring into another 100 cc. graduated flask enough of the stronger solution so that when the flask was filled to the mark each cc. would contain approximately 1 mg. of the substance. The flask was filled to the mark with purified petroleum ether, mixed, and well stoppered.

TABLE I.

Oxidation Values of Phospholipids of Tissues.

The values are expressed in cc. of 0.1 N dichromate solution per mg. of phospholipid.

	No. of determin- ations.	Average values.	Greatest variation from aver- age.	Average variation from aver- age.
			per cent	per cent
Cat liver lecithin.....	10	3.05	2.5	0.8
Beef " "	8	3.04	2.8	1.5
Cat muscle "	7	2.98	3.0	1.7
Egg lecithin.....	4	2.96	1.7	0.8
Beef liver cephalin.....	12	2.97	2.3	1.0
Cat " "	4	2.91	4.0	2.0
Cat muscle "	7	2.93	2.6	1.4

Oxidation.—Oleo-palmityl lecithin when oxidized completely should require oxygen according to the following equation, it being assumed that nitrogen is set free as such:



Potassium dichromate with sulfuric acid yields oxygen as follows:



From which it may be calculated that 1 mg. of the lecithin would require 3.11 cc. of 0.1 N dichromate solution for oxidation. For a similar cephalin the value would be 3.12. Other lecithins and cephalins would give only slightly different values and since oleic and palmitic acids are the acids found in largest amounts in these compounds in the animal body, the value 3.11 to 3.12 cc. of 0.1 N dichromate solution per mg. should represent close to the theoreti-

cal oxidation value of the phospholipids. Actual oxidation values on tissue phospholipids were obtained as is shown in Table I, on solutions made up as above and used directly without precipitation.

The average value obtained for lecithin was therefore 3.02 or 97 per cent of the theoretical and for cephalin 2.93 or 94 per cent and for both 2.975 or 95.5 per cent, which indicates that the oxidation had gone substantially to completeness according to the equation given. The lower values for cephalin are doubtless referable to the fact noted in earlier work (5), that cephalin as precipitated never has its theoretical content of fatty acids. The convenient factor 3 cc. of 0.1 N dichromate solution = 1 mg. of phospholipid is therefore used.

Isolation of Phospholipids from Tissue Extracts. Precipitation with Acetone and $MgCl_2$.—In the preparation of samples for analysis by the gross methods, the phospholipids are freed from other fatty substances by precipitation from their ether or petroleum ether solutions with excess of acetone, with or without $MgCl_2$. The same procedure has been found satisfactory for the micro determination, and it was soon found that the use of $MgCl_2$ was absolutely necessary to get complete precipitation of the small amounts used. A comparison of the values obtained directly on the petroleum ether standard solution and those obtained on the substance precipitated from petroleum ether by excess of acetone and a little $MgCl_2$ was made on various samples of which the following shows the nature of the results.

	By weight. mg.	Direct oxidation. mg.	Oxidation after precipitation. mg.
Cat liver phospholipid (average of 40 deter- minations in good agreement).	3.21	3.02 (94.2 per cent)	3.00 (93.5 per cent)

Influence of $MgCl_2$ on Oxidation.—It was noticed by Mr. H. D. McEwen of this laboratory that there was often an odor of chlorine in the oxidation flasks, and the fact became apparent that the $MgCl_2$ was a possible source of error in the oxidation procedure, since free chlorine would be liberated and a corresponding amount

of dichromate reduced, resulting in higher apparent values. It was possible to evaluate this factor and to show, by the following procedure, that the MgCl_2 present at the time of oxidation was confined to that soluble in the ether used to dissolve the phospholipid precipitate and therefore accounted for in the blank determination, and that the precipitate itself contained no appreciable amount of chlorine. Varying amounts of the standard phospholipid solution—2, 3, 4, 5 cc.—were taken and the phospholipid determined as in the method below, with a blank including the MgCl_2 . Values were obtained as follows, the results being calculated on the basis of 1 cc. of the standard phospholipid used.

Amount of solution.	Liver lecithin (cat).	Liver lecithin (beef).
cc.	mg. per cc.	mg. per cc.
2	0.83, 0.84	0.89, 0.85
3	0.83, 0.81, 0.82	0.87, 0.89
4	0.82	0.91, 0.89
5	0.82, 0.83	0.91, 0.91

These results show that the same values per cc. were obtained whether a large or small volume of standard was used and indicate that the blank includes whatever is necessary for correction due to the chloride present.

Solubility of Phospholipid in Acetone-Petroleum Ether.—The solubility of the phospholipids in the acetone-petroleum ether with MgCl_2 under the conditions of the precipitation was investigated in two ways: Method A, by making determinations of varying amounts of phospholipid and noting whether greater values were obtained with the larger amounts, and Method B, by examination of the acetone-petroleum ether residues. For this latter purpose acetone-petroleum ether residues from thirteen determinations each were collected, evaporated to dryness, the residues extracted with petroleum ether, and the phospholipid precipitated with acetone and MgCl_2 in the regular way. With regard to Method A the results given immediately above (for testing the effect of MgCl_2) show that no higher values were obtained with the larger amounts. In Method B of four trials no precipitates were obtained in two, and in the other two the precipi-

tates obtained when oxidized and calculated on the basis of a single precipitation gave in both cases a value of 0.02 cc. of 0.1 N dichromate, which is within the error of measurement. It may be concluded, then, that the amount of phospholipid soluble in the acetone-MgCl₂ solution is within the limit of error of the method and therefore negligible.

Procedure for Determination of Phospholipid in Blood and Tissues.
Extraction.

Whole Blood.—5 cc. of whole blood are run with continuous stirring, into a 100 cc. volumetric flask containing about 75 cc. of redistilled 95 per cent alcohol. The mixture is heated to boiling on the water bath and kept gently boiling with occasional stirring for 5 minutes, after which it is cooled, made up to volume with alcohol, and filtered through fat-free filter paper.

Plasma or Serum.—The procedure may be carried out as above for whole blood or as described earlier for plasma, as follows: 5 cc. of plasma are run, with continuous stirring, into a 100 cc. volumetric flask containing about 75 cc. of a mixture of 3 volumes of 95 per cent alcohol and 1 volume of ether, both redistilled. The mixture is heated to boiling on the water bath (with frequent stirring to avoid superheating), kept at the boiling temperature for a few seconds, then cooled to room temperature, made up to volume, and filtered through fat-free filter paper.

Corpuscles.—The extraction is made as for tissues (below).

Tissues.—The tissue, obtained either from the living animal or as soon as possible after death, is cut up fine with scissors into a watch-glass fitted with a short length of stirring rod and with another watch-glass as a cover. The whole is weighed to mg.; then about 1 gm. of the minced tissue is transferred by means of the stirring rod to a 4 inch mortar containing about 5 gm. of clean, sized sand (see below), and the watch-glass with cover and rod weighed again to obtain the exact weight of the tissue to be analyzed. The tissue (in the mortar) is ground with the pestle until the sand is reduced to a powder, care being taken to keep it mixed with the tissue; a small, narrow spatula (palette knife) is used for this purpose. The whole mass is then quantitatively transferred to a 100 cc. graduated flask as follows: The pasty mass is scraped together with the spatula and transferred in small

portions to the bottom of the flask, care being taken not to soil the neck, until all available material has been removed. 1 cc. of water is then added to the mortar and the grinding continued to loosen the adherent material, which is then stirred up and poured with the small portion of water into the flask. The washing and rubbing with 1 cc. of water are repeated twice more. At the end of the third washing the mortar and pestle should be clean and free from all but traces of tissue. The material in the flask is shaken with the water present until the large lumps are broken up. Redistilled alcohol is added to the volumetric flask until the volume is about 75 cc. The material in the flask is then heated to boiling on the water bath and kept boiling with stirring for 5 minutes, after which it is cooled to room temperature, made up to volume, and filtered.

Isolation of Phospholipids.—Aliquots of the extract containing about 2 mg. of phospholipid (of blood or plasma extract about 20 cc.; of tissue extracts, volumes approximately as follows: liver, kidney, pancreas, heart, about 10 cc., brain or nerve tissue 5 cc., striated muscle 15 cc.) are measured into small beakers and evaporated to dryness. The lipid of the residue in the beaker is dissolved by gently boiling with successive small portions (at least three) of petroleum ether, and decanting the liquid into 15 cc. graduated centrifuge tubes until the volume reaches about 10 cc. The extract is centrifuged to clear it, then transferred quantitatively to another set of graduated centrifuge tubes. The solvent is concentrated to 2 cc. in the centrifuge tubes by immersion in a beaker containing about 1 inch of hot water, a boiling tube (made by fusing about $\frac{1}{2}$ inch of melting point tube to the end of a 6 inch length of 2 mm. stirring rod) being used to prevent explosive boiling. To the 2 cc. of solvent in the centrifuge tube are added 7 cc. of redistilled acetone and 3 drops of a cold saturated solution of $MgCl_2$ in alcohol. The mixture is well stirred with a small stirring rod or by bubbling air through it, and the phospholipid precipitate separated by centrifugation at about 1500 R.P.M. for 5 minutes. At the end of the centrifugation, the acetone solution should be water-clear and is poured off from the phospholipid, which is now adherent to the bottom and walls of the tube. The precipitate is rinsed once with acetone, drained, and dissolved in 5 cc. of moist ether (made by shaking ordinary

peroxide-free ether with water until saturated)—dry ether does not dissolve it. Solution is sometimes slow and is aided by stirring with a length of small stirring rod. Ample time for solution must be allowed and generally there is very little that does not dissolve. The traces of undissolved residue together with the drop of watery $MgCl_2$ are separated by centrifugation for about 3 minutes at 1500 R.P.M., and the clear ether transferred quantitatively to the digestion flasks used for the oxidation without allowing the drop of watery liquid at the bottom to get out of the centrifuge tube. The transfer may be made by decanting or better by drawing the ether out of the centrifuge tube into the digestion flask with the help of a rubber bulb and a tightly fitting 2-hole stopper. The stopper fits the necks of the digestion flasks used and is fitted with (a) a short length of glass tubing to which the bulb is attached and (b) a length of glass tubing drawn out to a small opening, bent almost to U-shape and long enough so that the drawn out end will reach to the bottom of the centrifuge tube. The short free ends of both tubes project through the stopper. For use, the stopper is inserted tightly into the digestion flask, the bulb emptied of air by pressure, the tip of the long tube inserted into the ether in the centrifuge tube, and the bulb slowly released, when the ether will be drawn into the digestion flasks. The tubes are rinsed twice with 1 cc. more of ether, the washings being added to the main body of the ether. The solvent is removed by evaporation, the last traces being blown out with a gentle stream of air, the oxidation mixture added, and the oxidation carried out as below.

Oxidation.—The ether solutions in the digestion flasks are evaporated on the water bath and the last traces of solvent are blown out with a gentle stream of air. To the flasks are then added 5 cc. of the silver reagent (measured carefully because it contains some chromic acid) and then 3 cc. (accurately measured) of the N dichromate solution, with rotation. A control containing all the reagents or their residues except the fatty material is prepared and run along with the samples under exactly the same conditions. Ordinarily, a set consists of four samples and one control.

After the material is well mixed by rotation, the flasks are *loosely* stoppered and set in the oven at 124° . After 5 minutes heating

they are removed, rotated to stir up the contents, the stoppers *tightly* inserted, and the flasks replaced in the oven for the remainder of the heating period—a total of 15 to 20 minutes. At the end of this period the flasks are removed from the oven and without cooling 75 cc. of distilled water are added, the solutions cooled by gentle rotation in cold water after which the excess of dichromate is measured by titration as follows:

Titration.—To the flask are added 10 cc. of 10 per cent potassium iodide and without stirring (which might result in loss of iodine by fuming), the 0.1 N thiosulfate is run in from a burette, the contents of the flask being kept mixed by rotation, at first gentle, then as the iodine content diminishes more forceful. The silver causes a white precipitate to form which, however, does not interfere with the color change in the titration or with the end-point. When the titration is nearly complete, a few drops of 1 per cent starch solution are added and the titration is finished as usual. The titration of the blank is carried out in the same way. The difference between the titrations of the blank and the sample represents the amount of 0.1 N dichromate solution used by the fatty material in the sample.

Calculations.—Below is a typical calculation.

Weight of sample of tissue = 1 gm.

Aliquot taken, 10 cc. = 100 mg. of tissue.

Factor 3.00 cc. of 0.1 N dichromate = 1 mg. of phospholipid.

Titration of blank = 36.50 cc. of 0.1 N thiosulfate.

“ “ sample = 27.45 “ “ 0.1 “ “

Difference = 9.05 “ “ 0.1 “ “

Equivalent to 9.05 cc. of 0.1 N dichromate.

Representing $\frac{9.55}{3.00}$ = 3.02 mg. of phospholipid in 100 mg. of tissue.

The tissue contains 3.02 per cent of phospholipid.

Notes on Procedure.

Oxidation.—The digestion mixture should remain definitely brown throughout the heating period, indicating an excess of oxidizing agent. If the mixture becomes green or even shows a marked green tint either when the reagents are first mixed or at the end of the first 5 minutes of heating, it means that the necessary excess of oxidizer is not present. The determination may

ordinarily be saved by the addition of a second amount of silver reagent and dichromate and continuing the heating, but another determination should be run as a check with a sufficiently smaller aliquot to make sure that the necessary excess of reagent is present.

As in the method for lipids in blood (3), the oxidation may be carried out either in a hot air oven adjusted to 124°, fitted with a $\frac{3}{8}$ inch iron plate on which the flasks are set, or on the steam bath, with heating for 1 hour at 87–90°. The steam bath heating gives the same results as the oven heating and has the advantage that the time factor is not as critical,—the heating may be continued for a half hour overtime without affecting the results.

Correction.—As may be seen from the above, the method accounts for about 93 per cent of the *weight* of the known phospholipids taken. Although these have been purified in the standard way, there is little doubt but that they still contain some foreign material. It is, therefore, a question whether the missing 7 per cent is due to a fault in the method or to impurity in the standard substances, with the probability that both are responsible. For this reason a correction is not at present advised.

Extraction.—The preparation of the tissue for extraction by grinding with sand is an old procedure and offers the advantages of simplicity, ready availability of materials, and when well done, effectiveness. The sand used in this work was prepared by sizing—that which passed a 20 mesh but did not pass a 40 mesh sieve being used—and washing, by digesting with 10 per cent hydrochloric acid until there was no further effervescence or color, then with water to remove the acid, then boiling with 95 per cent alcohol until nothing further came away. The product was dried and kept in a closed flask. The measurement of the 5 gm. was made with a small test-tube with a mark on it to indicate approximately 5 gm.

The amount of water in 5 cc. of blood was taken as a guide to the amount to be used in rinsing out the mortar and the rinsing water therefore was limited to about 3 cc.

The extraction of the lipids from material prepared as above is practically complete as is shown by the following experiments. (a) The sand and tissue after extraction according to directions were rinsed out of the flasks onto a filter and washed three times with 25 cc. of cold alcohol. The whole was then extracted 8 hours in a continuous hot extractor with alcohol, the solvent removed

by evaporation, and the residue slightly acidified and extracted with petroleum ether. The lipid content of the petroleum ether was determined as given above. Four samples of the residue from 2 gm. of beef pancreas (containing 1.67 per cent of phospholipid) yielded amounts of lipid as follows (calculated as oleic acid): 0.35, 0.20, 0.23, 0.4 mg., average 0.14 mg. per gm. of tissue or 0.014 per cent. (b) The washed residues from 2 gm. samples of the gray matter from beef cerebellum (containing 5.3 per cent of phospholipid) were digested for 12 hours on the water bath with 20 per cent KOH, the mixture acidified, extracted with petroleum ether, the solvent removed, and the residue oxidized as usual, yielding values (as oleic acid) as follows: 0.22, 0.19, 0.41 mg., average 0.13 mg. per gm. of tissue or 0.013 per cent.

The extract contains all the lipids in the tissue and may be used for determination of total fatty acids and cholesterol with the procedure described for lipids in plasma (3).

Comparison of Methods.—The precipitation of phospholipid by acetone and $MgCl_2$ may, of course, be used preliminary to the determination of lecithin by its phosphorus content as in the earlier procedure (nephelometric or colorimetric) and has the advantage of limiting greatly the amount of non-phospholipid phosphorus determined. A series of comparative determinations of phospholipid was made by the three procedures: (a) oxidative as above, (b) nephelometric after precipitation with acetone and $MgCl_2$, (c) ordinary nephelometric on the alcohol extract. Results were obtained as shown in Table II.

The results indicate that with blood plasma the oxidative and ordinary nephelometric methods agree quite well. In tissue extracts the ordinary nephelometric method generally gives higher, sometimes much higher results than the oxidative or the precipitation nephelometric procedures. The oxidative and the precipitation nephelometric methods generally agree quite well with each other.

Peroxides in Ether.—The presence of peroxide in the ether used is a source of considerable trouble, partly because it remains behind in the digestion flasks after the ether has gone, and partly from its unknown effect on the phospholipids. All ether used should be tested for peroxide, by shaking with 0.5 volume of slightly acidified (H_2SO_4) 10 per cent potassium iodide. If more

TABLE II.

Comparison of Methods for Phospholipid in Blood and Tissues.

The results are expressed in mg. of phospholipid per 100 gm. of material.

Samples.	Oxidative.	Nephelometric after precipi- tation.	Nephelome- tric,* ordi- nary.
Diabetic dog plasma.	410		398
	410		429
	432		450
	400		395
	431		450
	415		400
	510		486
	465		439
	445		429
Plasma from epileptic children.	502		488
	425		464
	142		156
	147		147
	167		178
	186	170	294
	287	244	
Beef liver.	4760	4200	4950
Rat "	2280	2270	2390
" kidney.	2150	2120	2100
" testicle.	1550	1560	1900
" muscle.	1180	1300	1390
Dog liver.	2230	2300	3180
Beef "	4250		4310
" "	3960		4050
" cerebellum (gray matter).	5300		4740
" medulla.	10400		8800
Frog muscle.	770	720	1060
" skin.	1010	1000	1970
" liver.	1150	1100	2000
" egg mass.	3450	3350	4440

* The nephelometric determinations on diabetic plasma were made by Mr. H. D. McEwen of this laboratory.

than a faint yellow color is obtained, the sample should either be rejected or treated to remove the peroxide. The following procedures for removal of peroxide were tried and found fairly effective: (a) Distillation with a short fractioning column. Since the peroxide has a higher boiling point than the ether, most of it remains behind. Only the first two-thirds of the ether which distils over should be used. (b) Washing, by shaking with acidified 10 per cent potassium iodide, sodium thiosulfate solution being added to prevent the accumulation of excess of free iodine. The shaking and decolorization should be kept up until no further color is obtained. The ether is then washed with water, distilled, and kept in dark bottles or in the dark. If ether containing peroxide is used, the peroxide will reveal itself by its acrid odor in the digestion flasks after the ether has been removed. In this case the heating and blowing out must be continued till all the peroxide odor is gone; otherwise too high values will be obtained.

Reagents Used.—0.1 N sodium thiosulfate, N potassium dichromate, 1 per cent starch solution, 10 per cent potassium iodide.

Sulfuric Acid Reagent.—Pure concentrated sulfuric acid containing silver dichromate (silver reagent) prepared after Nicloux (6) as follows: To 5 gm. of silver nitrate dissolved in 25 cc. of water in a 100 cc. centrifuge tube were added 5 gm. of potassium dichromate dissolved in about 50 cc. of water. The precipitated silver dichromate was separated by centrifugation, washed twice by centrifugation with water to get rid of the nitric acid, and the cake of precipitate dissolved without drying in 500 cc. of pure concentrated sulfuric acid with the aid of gentle heating.

Petroleum Ether.—Commercial petroleum ether was fractionally distilled with a Clarke's column, that which distilled above 60° being rejected. The distillate below 60° was washed with concentrated sulfuric acid and redistilled with an ordinary condenser. 50 cc. portions of this purified petroleum ether gave no blank when carried through the procedure below. Fair results can be obtained with unpurified commercial petroleum ether, provided sufficient care is taken to remove the last traces of solvent from the fatty substances before oxidation (a difficult operation) and adequate control determinations are made, but the purified material is well worth the effort of preparing it.

Ether.—Peroxide-free ether, moistened by shaking with water, kept in a dark bottle with a small excess of water.

Acetone.—Commercial acetone, once purified by distillation.

MgCl₂.—Cold-saturated solution in 95 per cent alcohol.

For the oxidation, glass-stoppered, Erlenmeyer flasks of 125 to 150 cc. capacity are required. These are cleaned for use by treatment with cleaning mixture (concentrated sulfuric acid and dichromate) for 1 hour in the hot oven at 124°, then rinsed well with water, dried with heat, and kept stoppered.

Heating Apparatus.—(a) An electrically heated hot air oven adjusted to 124°, with temperature adjustment accurate to $\pm 2^\circ$. The ordinary inexpensive hot air ovens of several makes were found satisfactory. These were fitted with a special shelf consisting of a $\frac{3}{8}$ inch iron plate. The ovens should be heated for at least 2 hours before use in order to come to constant temperature, and the temperature should be checked before each set of determinations. (b) A water or steam bath which will maintain a temperature of 88–90°. The holes in the bath in which the flasks rest should be just large enough to hold the flask without allowing it to slip through.

Temperature Control.—The temperature is in each case to be measured by the use of a thermometer suspended in the liquid in a flask similar to the ones used for the determinations, the liquid being a measured sample of the mixture as used in the oxidations. The thermometer should be compared with a standard thermometer at the temperature used. Since the time-temperature factor is critical, these details should be closely checked.

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VITAMIN REQUIREMENTS OF NURSING YOUNG.

VI. ANHYDREMIA ASSOCIATED WITH DISTURBANCE IN HEMATOPOIETIC FUNCTION IN NURSING YOUNG OF THE ALBINO RAT SUFFERING FROM A DEFICIENCY OF THE VITAMIN B COMPLEX.*†

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In attempting to study biochemical changes in the blood of nursing young of the albino rat we at once encountered an apparent handicap in obtaining sufficient blood. After a number of preliminary trials we found, however, that by clipping the tail of the baby rat sufficient blood could be secured for a hemoglobin determination and an erythrocyte count. Later we determined that a nursing weighing 18 to 25 gm. could in addition furnish enough blood for a determination of the refractive index of the blood serum without any accompanying loss of weight of the animal. By sacrificing the animals we could secure additional information by making determinations of the total blood solids. We thus found a procedure of studying the effect of vitamin deficiencies in the maternal diet on the hematopoietic function of baby rats. In this paper we are reporting on the results of our first investigation; namely, anhydremia associated with disturbance in hematopoietic function of nursing young of the albino rat suffering from a deficiency of the vitamin B complex.†

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† The term "vitamin B complex" is used in this paper to designate a combination of the labile and stable factors, provisionally termed vitamins B and F respectively in a recent communication (*J. Biol. Chem.*, **80**, 306 (1928)).

The technique employed is essentially that of Hart and co-workers (1). Hemoglobin determinations were made according to the method of Newcomer (2) on a Bausch and Lomb colorim-

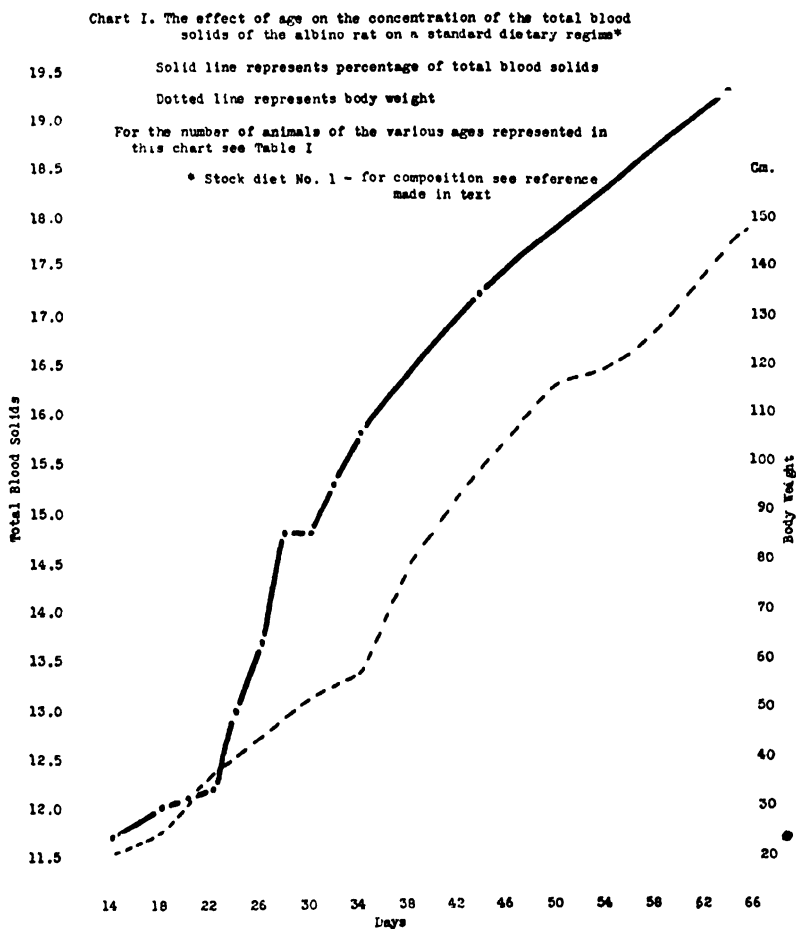


CHART I.

eter with a standardized colored disc, which served as a hemoglobinometer attachment. The accuracy [of the colorimetric readings checked with Van Slyke and Neill's oxygen capacity method (3) to the extent of ± 3 per cent. For the erythrocyte

counts we used a Levy hemocytometer with single Neubauer ruling. A number of experiments on control nurslings has revealed no appreciable differences between results secured from peripheral and arterial blood. The results of our findings are presented in Tables I to III and in Charts I to VII.

TABLE I.

Number of Animals of Different Ages Employed for Hemoglobin Determinations, Erythrocyte Counts, and Determinations of Total Blood Solids, Represented in Charts I and II.

Age.	No. of Hb determinations and erythrocyte counts.	Average weight of each animal.	No. of determinations of total blood solids.	Average weight of each animal.
<i>days</i>		<i>gm.</i>		<i>gm.</i>
14	17	22	3	21
15	15	22	3	22
16	27	23	18	22
18	54	27	11	26
19	30	28	12	28
20	29	28	14	28
21	30	33	15	33
22	27	38	9	37
23	34	39	15	40
24	24	40	11	41
26	27	44	10	45
28	26	50	14	50
30	36	53	10	53
32	27	56	14	55
34	23	54	17	57
38	23	82		
44	24	100	13	98
50	23	125		
63	24	149	10	135
Total.....	520		199	

For control animals we have used nursing young from our colony receiving Stock Diet 1 (4) and, since in a great proportion of our pathological nurslings we have developed prolonged maintenance during lactation, we have secured additional data on hematopoietic function of control animals during the post lactation period extending to the 64th day of life. Chart I shows the effect of age and body weight on the concentration of total blood

solids in the albino rat from the 14th to the 66th day. For this work, as indicated in Table I, we have employed a total of 199

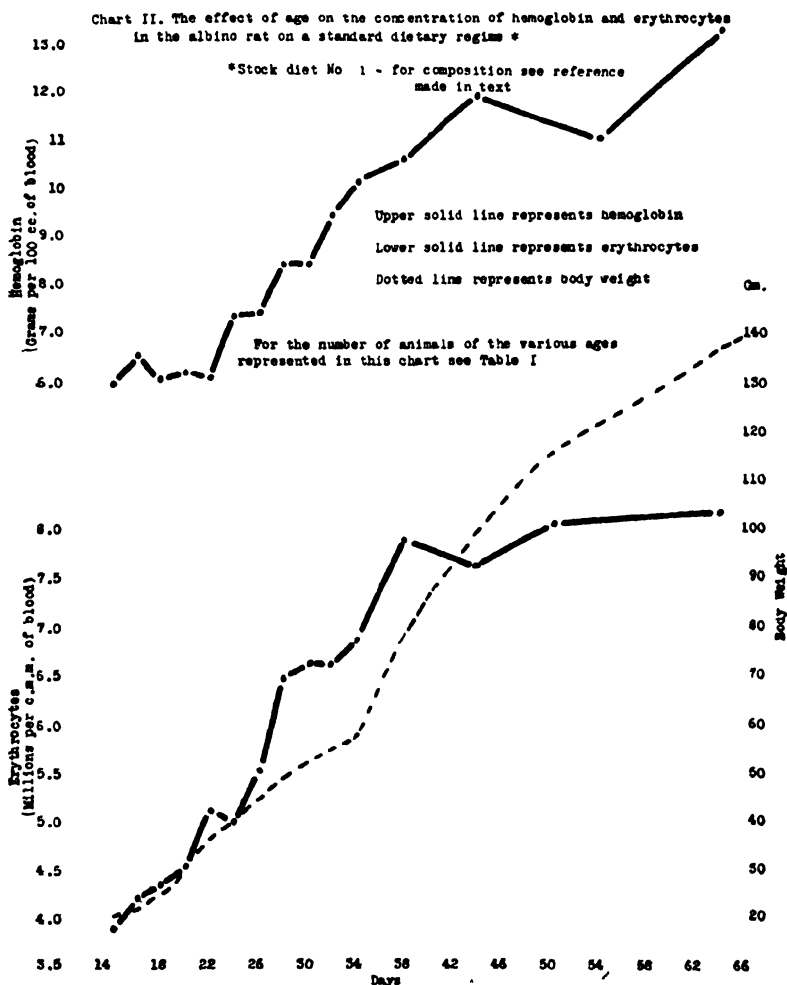


CHART II.

animals. In Chart II we are showing graphically the effect of age and body weight on the concentration of hemoglobin and erythrocytes covering a period of 64 days on our standard dietary régime

(Stock Diet 1). For the latter work we have made 520 determinations.

For our pathological work we have employed a total of 125 nursing young. The various stages of the avitaminosis were produced by several different modifications of biological procedures. First we produced the polyneuritic condition in the nurslings, accompanied by posterior paralysis, muscle chills, and convulsions according to the technique previously described by transferring females with litters on the date of birth of the young to vitamin B-deficient Ration 1009 (5). During the 1st day of maintenance of the young we generally encountered only the incipient stages of paralysis. On the following day the spastic condition was frequently accompanied by convulsions and, if the nurslings survived the convulsive state, we were afforded the opportunity of observing the terminal stages of beriberi. By allowing the lactating mother at the plateau curve of the nursing young (indicating depletion of storage of vitamin reserves of the mother) daily suboptimum amounts of either dehydrated yeast or yeast concentrates for lactation, prolonged maintenance of the nursing young was produced. The daily allowance could be determined only after several preliminary trials. Later, we found another means of producing prolonged maintenance in the nurslings; *i.e.*, by the daily administration of one of our less concentrated yeast extracts to the young in such dosages that would prevent growth and loss of body weight. Chart VII shows a representative case. For brevity we are deleting the daily weights of the mother and the daily food consumption records, and are showing only the growth of the litter. The term "maintenance" is used to signify relatively small changes in body weight during long periods of lactation when the nursing young should make rapid growth. When a plateau curve is produced in a group of young (litter of six) some individuals may be actually gaining several gm. and others losing to an equal extent. Such has been the case with the litter represented in Chart VII. The point of interest in this paper is the pronounced biochemical changes produced in the blood with relatively insignificant accompanying changes in body weight.

The work on our pathological animals may be divided into two parts, as follows: (1) The development of polyneuritis in the nursing young in various stages of the disease. Hemoglobin deter-

minations and erythrocyte counts were made on various groups of nurslings, blood being secured by peripheral bleeding. The young were then anesthetized with chloroform and enough blood was obtained from the carotid artery for the determination of total blood solids. A total of 62 animals was used for this phase of our investigation. Similar technique was employed in securing data on our control animals. (2) The production of prolonged maintenance in the nursing young without the accompaniment of any apparent external pathological symptoms with the exception of incipient posterior paralysis in some cases. The nurslings were bled several times a week peripherally, and enough blood was secured for hemoglobin determinations, erythrocyte counts, and determinations of the refractive index of the blood serum. The latter determination showed the per cent of serum proteins and served as an index of the concentration of the blood (6). In calculating the per cent of serum proteins from the refractive index figures we applied the following formula of Reiss (6).

$$\frac{R - R_1 - 0.0022}{0.00172} = \text{per cent of serum proteins}$$

R = refractive index of the serum at 20°.

R_1 = " " " distilled water (1.3326 at 20°).

0.0022 = correction for non-protein constituents of the blood.

0.00172 = refractive index of 1 per cent protein of blood serum.

The hematopoietic function of 63 nursing young was studied for a period of 3 to 6 weeks in the prolonged maintenance state during lactation, the main purpose being to note changes in the blood as evidenced by concentration of the above mentioned constituents.

On our Stock Diet 1 nursing young reach a weight of 40 gm. each on about the 24th day of lactation when they are considered weaned. On the 20th day nurslings whose mothers receive the same diet reach a weight of 28 gm. each. Table II shows the hematopoietic function of nursing young of the albino rat during various stages of polyneuritis. In this table we are submitting some typical cases and are showing maximum, minimum, and average results.

The incipient stage is characterized by posterior paralysis, accompanied by cessation of growth, and in some cases, slight loss of body weight. In that condition concentration of blood is

apparent when a comparison is made between the per cent of total blood solids of these young with normal young of the same age and body weight (Chart I). The control animals show a concentration of 11.5 to 12.0 per cent, while the pathological group, a con-

TABLE II.

Hematopoietic Function of Nursing Young of the Albino Rat during Various Stages of Polyneuritis.

Age.	Nursling No.	Weight of nursling.	Hb	Erythrocytes.	Total blood solids.	Stage of avitaminosis.
<i>days</i>		<i>gm.</i>	<i>gm. per 100 cc.</i>	<i>million per c.mm.</i>	<i>per cent</i>	
20	4979-a	20.0	6.76	5.88	14.5	Incipient.
20	4979-c	18.0	8.30	5.68	14.3	"
20	4979-e	16.0	6.76	4.92	16.8	"
20	4905-f	19.0	6.46	2.60	15.0	"
	Average for 11 nurslings.	18.4	6.86	4.60	15.3	"
23	4904-a	14.0	8.48	6.33	19.2	"
23	4904-b	16.0	7.26	5.89	15.6	"
23	4904-f	16.0	8.61	2.42	16.3	"
	Average for 6 nurslings.	16.5	7.86	5.16	16.7	"
16	Average for 6 nurslings.	17.6	7.04	4.51	13.8	Convulsive.
24	" "	23.6	5.98	5.61	13.9	"
20	5016-c*	14.0	7.20	4.48	21.6	Terminal.
24	4916-b*	29.0	12.61	6.14	21.4	"
24	4916-c*	30.0	14.58	6.34	21.9	"
24	4916-d*	29.0	5.37	5.51	19.5	"
24	4916-e†	32.0	10.56	4.86	14.2	"
24	4916-f†	34.0	6.46	5.70	21.1	"
	Average for 5 nurslings, 24 days old.	30.0	9.91	5.71	19.6	"

* In dying condition; bled from carotid artery.

† Bled peripherally.

centration of 15.35 to 16.7 per cent. The control animals show a concentration of 6 gm. of hemoglobin per 100 cc. of blood, and an erythrocyte count of 4.5 millions per c.mm., while the pathological group shows considerably higher values for both hemoglobin and

red blood corpuscles. Out of seventeen individuals in the incipient stage of the avitaminosis, sixteen showed concentration of hemoglobin, and thirteen concentration of erythrocytes. Four individuals showed low erythrocyte counts, two below 4 millions per c.mm., and two below 3 millions per c.mm.

All the twelve nurslings in the convulsive state of polyneuritis, characterized by screaming, running fits, indicate concentration of total blood solids, but not to the same extent as the group in the incipient stage of polyneuritis. Such results were surprising to us, since we have throughout our work considered the convulsive state to be a more advanced pathological symptom associated with the avitaminosis produced by a deficiency of the vitamin B complex. The disturbance in hematopoietic function of this group of pathological nursing young is also evident. The 16 day old group shows concentration of hemoglobin, while the 24 day old group shows concentration of erythrocytes.

The most pronounced concentration of blood is encountered in the terminal stage of polyneuritis, characterized by posterior paralysis, chills, shallow and intermittent respiration, and cyanosis. The average concentration of total blood solids for five nurslings in the terminal stage of the avitaminosis is 19.6 per cent, or an increase in concentration of 55 per cent. On our Stock Diet 1 a concentration of 19.6 per cent of total blood solids is not reached until the 64th day of life when the body weight is approximately 150 gm. Increase of concentration of hemoglobin and erythrocytes is also quite marked.

It may be argued that with the onset of the avitaminosis there is a considerable constriction in the capillaries and peripheral blood vessels, which would necessarily yield blood of a higher concentration than that drawn from a vein or an artery. Since four nurslings, shown in Table II, were in a dying condition, we were obliged to secure blood from an artery and in such cases we found the most convenient channel the carotid artery. It will be noted that the arterial blood is, nevertheless, highly concentrated. We have also found blood drawn from the femoral vein of a half dozen pathological animals to be concentrated.

In order to follow the blood picture in nursing young suffering from a deficiency of the vitamin B complex during lactation, we had to modify the biological technique so as to prevent a crisis

soon after the depletion of the vitamin reserves of the lactating mother. Since we were desirous of studying the concentration of the serum proteins as well as the concentration of hemoglobin and erythrocytes, rapid loss of body weight had to be circumvented. Loss of body weight may be accompanied by rapid catabolism of all blood as well as tissue constituents, and such complication would yield values difficult to interpret in so far as blood concentration is concerned. We also attempted to keep the weight of the pathological nurslings at a maintenance level, because even slight gains in weight are followed by increases in the refractive index of the blood serum. In the majority of trials we have been able to produce prolonged maintenance in the nursing young according to the technique described in Chart VII. The vitamin B extract administered to most of the nursing young in daily amounts for this purpose was prepared by fractional precipitation with alcohol. This preparation was designated as Yeast Concentrate 26.¹

For a comparison of the effect of age and body weight on the hematopoietic function we have studied a group of animals receiving a diet (abundant in the vitamin B complex) of the following composition: casein (purified) 20, Northwestern dehydrated yeast 10, McCollum's salt mixture (7) 4, butter fat 5, and dextrin 61. In most of the animals of this group we have determined the influence of age and body weight on the concentration of serum proteins as well as the concentration of hemoglobin and erythrocytes. Chart III shows our results graphically, representing the average of 286 hemoglobin determinations, 245 erythrocyte counts, and 240 determinations of serum proteins (see Table III).

By comparing the influence of age and body weight on the concentration of hemoglobin and erythrocytes of animals on the two types of diets, Stock Diet 1 and synthetic Ration 1145 (Charts II and III), it is apparent that the latter diet, containing an abundance of dehydrated yeast, stimulates production of a greater concentration of hemoglobin not only during the later part of lactation but also during the period of growth following weaning. Between the 14th and 22nd day of the nursing period the concen-

¹ Prepared by E. H. Stuart, Eli Lilly Research Laboratories, Indianapolis.

tration of hemoglobin on synthetic Diet 1145 is between 8 and 9 gm. per 100 cc. of blood, while on Stock Diet 1 the concentration is only 6 per cent. Between the 21st and 26th day there is also an

Chart III. The effect of age on hematopoietic function of the albino rat on a synthetic ration* containing 10 per cent dehydrated yeast (* for composition of ration see text)

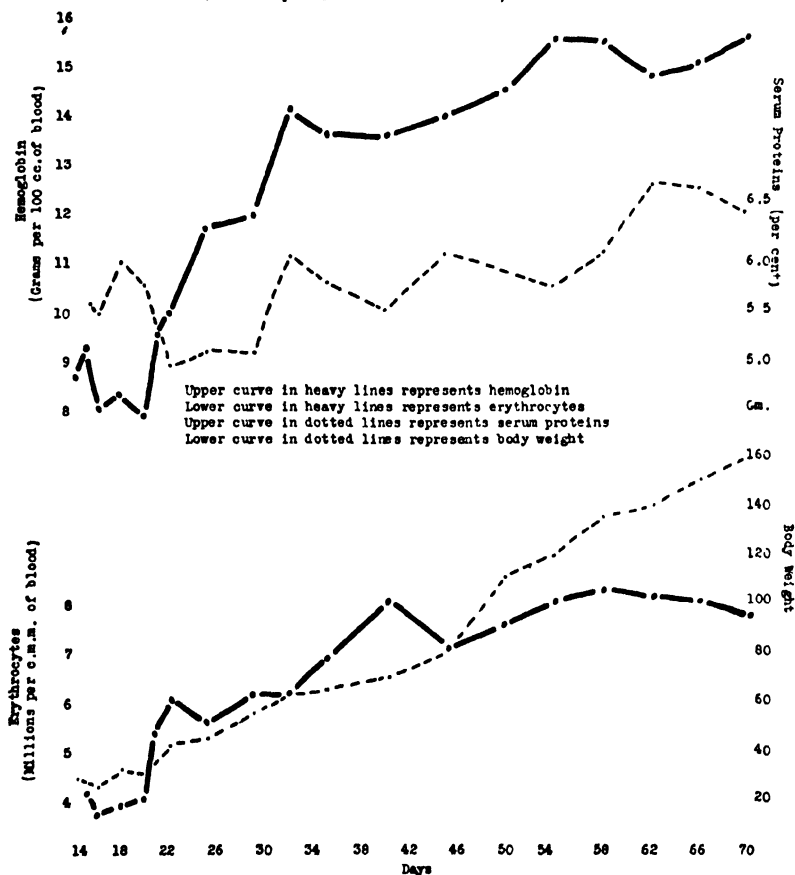


CHART III.

appreciably greater concentration of red blood corpuscles on the synthetic ration than on the stock cereal diet. After that period there is no significant difference in the concentration of erythrocytes on the two types of diets. The concentration of hemo-

globin, however, is considerably greater on the yeast-containing ration throughout the rest of the experimental period.

The question now arises: Which curve of hematopoietic function should we consider as the control, that represented by animals

TABLE III.

*Number of Animals of Different Ages Employed for Hemoglobin Determinations, Erythrocyte Counts, and Determinations of Serum Proteins, Represented in Chart III.**

Age.	Average weight of each animal.	No. of determinations for:		
		Hb	Erythrocytes.	Serum proteins.
<i>days</i>	<i>gm.</i>			
14	27.0	6		
15	27.8	12	12	12
16	25.0	3	3	3
18	32.0	6	6	6
20	31.0	6	6	6
21	36.0	12	12	12
22	41.5	11	11	6
25	44.5	23	12	12
29	55.0	18	12	12
32	63.5	22	15	15
35	65.0	23	18	18
40	71.0	12	6	6
45	85.0	24	24	24
50	110.0	18	18	18
54	120.0	18	18	18
58	135.0	18	18	18
62	140.0	18	18	18
66	150.0	18	18	18
70	160.0	18	18	18
Total		286	245	240

* Part of these data was accumulated with the assistance of Margaret Elizabeth Smith of the Department of Home Economics, University of Arkansas.

that subsisted on what we considered for the past 9 years an optimum ration; namely, Stock Diet 1 (4), containing an abundance of whole cereals, casein, cod liver oil, fortified with such minerals as NaCl and CaCO₃, and supplemented with a liberal daily allowance of fresh milk; or a purified ration containing an

abundance of the vitamin B complex in the form of dehydrated yeast? The greater concentration of hemoglobin in nursing young whose maternal diet consisted of synthetic Ration 1145 compared with those whose mothers received Stock Diet 1 may be attributable to greater growth in the former. Since Ration 1145 is abnormally high in vitamin B, our stock cereal diet would be more comparable with an optimum diet used by man, and we thought it reasonable, therefore, to make comparisons with the latter diet. Moreover, since the essential difference in the two diets is the greater amounts of the vitamin B complex furnished by the abundance of yeast in Ration 1145, and if the production of a greater concentration of hemoglobin on that diet is to be ascribed to the physiological action of vitamins B and F contained therein, then it follows that pathological nurslings on a maternal ration entirely deficient in these vitamins should have a considerably lower hemoglobin concentration than nursing young whose maternal diet (Stock Diet 1) stands intermediate between Ration 1145 and our ration deficient in the vitamin B complex (Ration 1009). But, on the other hand, the pathological nurslings show a greater concentration of hemoglobin than nursing young raised on Stock Diet 1, which demonstrates that the blood of the polyneuritic nursing young is undoubtedly concentrated. Chart III also shows the effect of age and body weight on the concentration of serum proteins. Between the 18th and 22nd day of lactation there is a drop in the concentration of serum proteins from 6 to 5 per cent and the latter concentration is maintained until the 30th day. From the 30th day on the concentration rises to 6 per cent and fluctuates between 5.5 and 6.0 per cent until the 54th day. It is of interest to note that between the 32nd and 54th day, while the body weight has increased from 63 to 120 gm., or an increase of 90.5 per cent, the serum proteins have been maintained at practically the same level. From the 54th day on there is a progressive rise to 6.5 per cent. These data have been secured on Ration 1145. Unfortunately, we have no such data on Stock Diet 1 to make a comparison on the effect of these two types of diets on the concentration of serum proteins.

Space does not permit us to show our results secured on 63 pathological animals. We are, therefore, showing graphically the hematopoietic picture of only two cases. Charts IV and V

show that, although we may encounter slight increases in body weight of nursing young and the nurslings may show no apparent external signs of abnormality, the blood in such individuals

Chart IV. Hematopoietic function of a nursling of the albino rat suffering from a deficiency of the vitamin B complex during the stage of prolonged maintenance. Maintenance in the nursing young was produced by the administration of a controlled daily dosage of a vitamin preparation (See Chart VII)

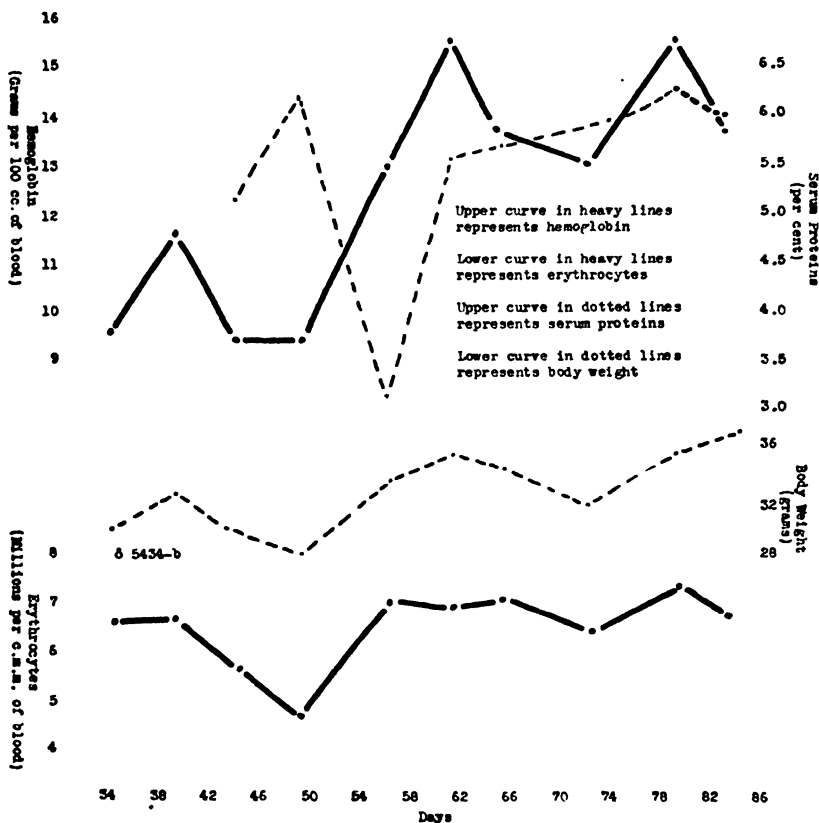


CHART IV.

shows pronounced biochemical changes indicating pathological symptoms, as evidenced by fluctuations and concentration of serum proteins, and by concentration of hemoglobin and erythrocytes. Nursling 5434-b (Chart IV) shows an increase in

hemoglobin concentration between the 49th and 61st day of 66 per cent and an increase in body weight of only 29 per cent. Between the 34th and 49th day the same individual shows a change in

Chart V. Hematopoietic function of a nursing of the albino rat suffering from a deficiency of the vitamin B complex during the stage of prolonged maintenance. Maintenance in the nursing young was produced by the administration of a controlled daily dosage of a vitamin preparation (See Chart VII)

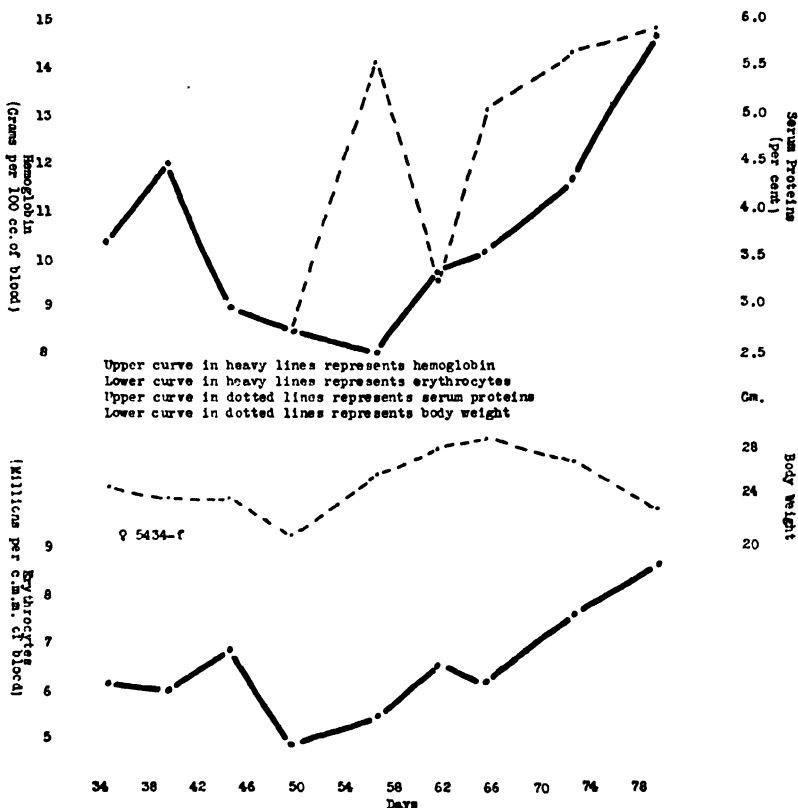


CHART V.

body weight of only 7 per cent and during the same period a change in the concentration of erythrocytes of 30 per cent. The sudden drop in the concentration of serum proteins between the 50th and 56th day during which time the hemoglobin increased is difficult to explain. The subsequent increase in the concentra-

tion of serum proteins and the maintenance of such a level of concentration, the high hemoglobin percentage shown by a rat weighing less than 40 gm., together with our findings of 18.4 per cent total blood solids on the 83rd day when the animal was sacrificed, conclusively demonstrate a loss of water from the blood. This individual, then, gives us a typical case of anhydremia with insignificant accompanying changes in body weight.

Chart V presents another illustration of hematopoietic changes in a nursling showing slight changes of body weight during lactation. Nursling 5434-f for a period of 44 days (with some intermediate fluctuations) has practically maintained its 25 gm. weight. The first drop in hemoglobin and red blood corpuscles suggests an anemia and the subsequent marked rises in both hemoglobin and erythrocytes, together with the increases in concentration of serum proteins and the finding of 17.8 per cent of total blood solids on the 79th day, when the animal was sacrificed, demonstrate anhydremia in this individual, although the changes in its body weight are relatively insignificant. The sudden drop in the concentration of serum proteins between the 56th and 61st day, as in the case of Nursling 5434-b (Chart IV) is again difficult to explain. That there is a change in the water balance of the blood is unquestionable. Perhaps this represents a temporary recovery period, associated with dilution of blood, since during that time the daily dosage of our less concentrated vitamin B preparation evidently was producing some growth. The increase in concentration of hemoglobin and erythrocytes during that period can be explained on the possibility that growth had a greater effect than dilution on blood concentration.

Reiss (6) has made an extensive investigation of blood concentration in disease, using the refractive index of the blood serum as a criterion of water balance. For instance, he demonstrated that in chronic nephritis with edema the blood is more diluted than in any other disease. Marriott (8), applying Reiss' chemical method of studying concentration of blood, found that in acute diarrhea of infants the first change noted is a marked increase of protein concentration, which may be as high as 10 to 12 per cent, as contrasted with a normal of from 6 to 8 per cent. Our results are in agreement with the recent findings of Darrow and Buckman (9) that the refractive index of the blood serum alone does not

always reveal the true picture of the water content of the blood in disease. This is particularly true when the catabolism has

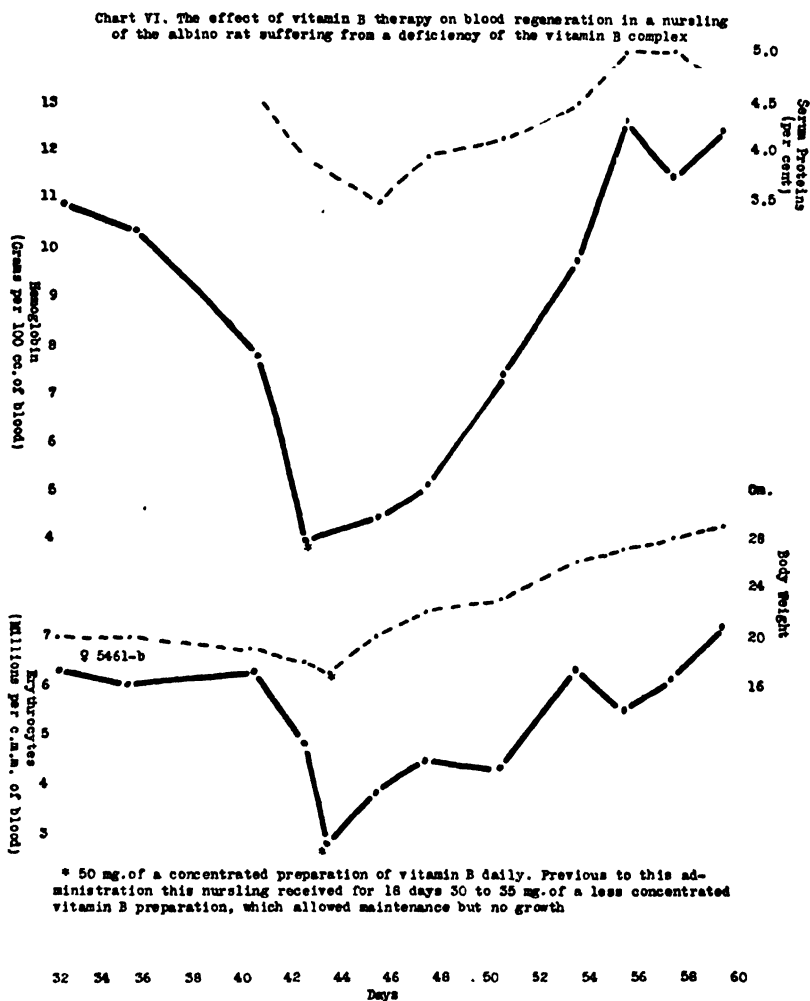


CHART VI.

progressed to the extent that there is a breaking down of serum proteins. Two conflicting factors then arise. The loss of water, on the one hand, increases the refractive index, and the breaking

down of the serum proteins, on the other hand, decreases that index. When the refractive index is taken in conjunction with determinations of other blood constituents, such as hemoglobin, erythrocytes, and total blood solids, it furnishes additional information on blood concentration. We further maintain, however, that when a period of prolonged maintenance of body weight is accompanied by pronounced increases of the concentration of serum proteins, even in the absence of other data, we have an unmistakable diagnosis of anhydremia.

Out of the 125 pathological nursing young investigated we found only sixteen that showed pronounced anemia as evidenced by low hemoglobin and erythrocyte counts. Vitamin B therapy was attempted on six individuals with marked success. Chart VI offers an illustration. During the period of 11 days of virtual maintenance, there being a loss of only 2 gm. of body weight during that period, Nursling 5461-b reduced its hemoglobin from 11 to 4 gm. per 100 cc. of blood, and its erythrocyte count from 6.5 to 2.8 millions per c.mm. of blood. On the 43rd day the nursling was allowed a daily administration of 50 mg. of a potent yeast concentrate used successfully in numerous previous trials (10).² The prompt response in regeneration of both hemoglobin and red blood corpuscles is evident. During the subsequent 15 days of vitamin B therapy this nursling, while gaining 55 per cent in body weight, increased its hemoglobin concentration 200 per cent, and its concentration of red blood corpuscles 157 per cent. Whether or not our yeast extracts carry a supplementary inorganic factor for blood regeneration has so far been impossible to demonstrate, since we have been unable to free our vitamin B extracts from inorganic matter, and the nurslings in the anemic condition develop a crisis and die, unless vitamin therapy is readily instituted. Several trials of supplementary iron administrations resulted in failure. In connection with the inorganic problem we have previously demonstrated that copper does not supplement our yeast extracts for growth (11). The first drop in the concentration of serum proteins following vitamin B therapy

² Previous to this administration this nursing young received for 18 days 30 to 35 mg. of a less concentrated vitamin B preparation, which allowed maintenance but no growth.

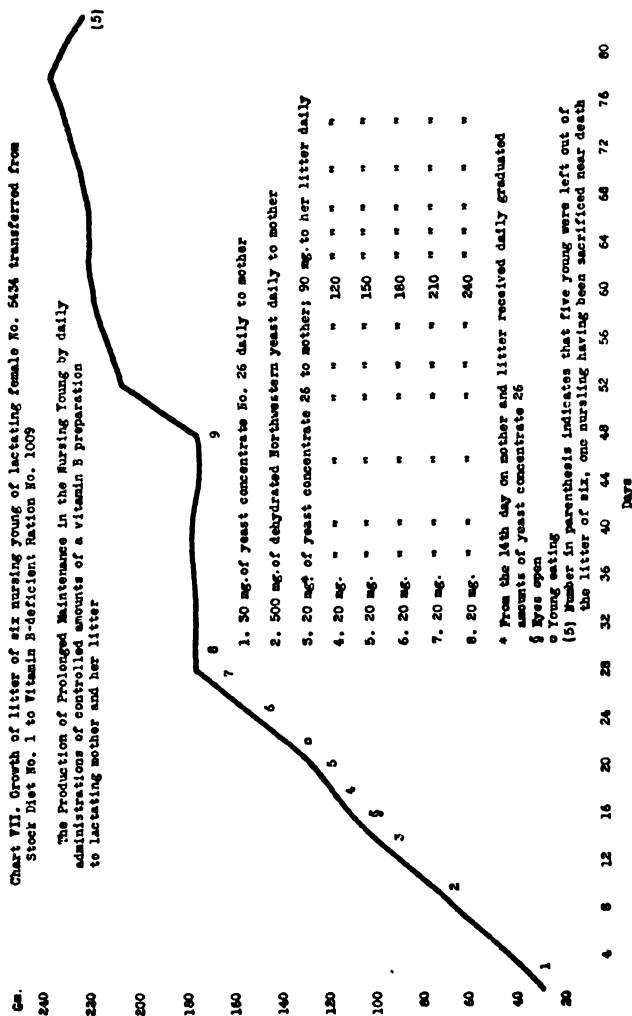


CHART VII.

probably represents the first recovery period accompanied by dilution of blood. The subsequent rise may be explained on the basis of growth.

The hematopoietic response to administration of vitamin B products, either in the form of natural foods, or highly concentrated preparations, can at least partly be ascribed to the influence vitamin B³ has in controlling food consumption. Lactating mother rats on a diet abundant in vitamin B eat approximately twice as much as lactating females on a diet entirely deficient in this syndrome; also nursing young during the later periods of lactation, after vitamin B therapy, partake more of the maternal diet. On our dietary régime partly deficient in the vitamin B complex, which produces prolonged maintenance in the nursing young during advanced stages of lactation, the daily food consumption of mothers and young is approximately 50 per cent less than on our rations providing an abundance of this dietary complex. The pronounced anhydremia associated with marked disturbance in hematopoietic function is, therefore, produced in a state of the avitaminosis long before complete anorexia resulting in starvation has set in. The entire regulatory mechanism by which the vitamin B complex produces anhydremia associated with disturbance in hematopoietic function is not as yet fully understood, but it is suggested that its controlling influence on food consumption may be a determining factor. That anhydremia is produced in nursing young of the albino rat suffering from uncomplicated vitamin B deficiency is demonstrated in the paper following.

SUMMARY.

Nursing young of the albino rat suffering from a deficiency of the vitamin B complex develop anhydremia, as is evidenced from hemoglobin determinations, erythrocyte counts, determinations of total blood solids, and studies of the refractive index of the blood serum. Such young also show marked disturbances in hematopoietic function.

* "Vitamin B" is used in the sense of the "vitamin B complex" in this paper.

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EFFECT OF VITAMIN DEFICIENCIES ON CARBOHY- DRATE METABOLISM.

I. HYPOGLYCEMIA ASSOCIATED WITH ANHYDREMIA AND DIS- TURBANCE IN HEMATOPOIETIC FUNCTION IN NURSING YOUNG OF THE ALBINO RAT SUFFERING FROM UNCOMPLICATED VITAMIN B DEFICIENCY.*

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(Received for publication, February 5, 1929.)

The purpose of this investigation was to study the concentration of blood sugar in nursing young of the albino rat suffering from uncomplicated vitamin B deficiency in the state of prolonged maintenance. The details of biological technique for the production of such avitaminosis will appear elsewhere, but essentially it consists of the administration to the lactating mothers of a diet, containing an abundance of vitamin F¹ in the form of auto-claved yeast, but deficient in vitamin B. Small daily allowances of dehydrated yeast during the early period of lactation, together with the storage of vitamin B from the previous diet, insure prolonged maintenance but no growth in the nursing young during the later periods of lactation. The nurslings eventually develop posterior paralysis, labored respiration, cyanosis, and finally death occurs, unless vitamin therapy is instituted.

In this investigation we have studied forty-four pathological nursing young suffering from uncomplicated vitamin B deficiency and six nurslings suffering from a deficiency of vitamins B and F (vitamin B complex). As controls we have studied forty animals on Ration 1145 (containing an abundance of vitamins B and F)

* Research paper No. 93, Journal Series, University of Arkansas. Supported by Purnell Funds.

¹ The term "vitamin F" refers to the stable antipellagric factor, according to a nomenclature suggested in a previous communication (1).

(2), and six young on Stock Diet 1 (3). In our preliminary work we have found the same degree of variations among individual animals on the two types of diets; therefore, we have taken most of our control determinations on rats receiving Ration 1145, such animals having been at that time available for this work. The blood sugar concentration was studied in all the 50 pathological animals. In thirty-nine of these we have in addition followed the changes in the concentration of hemoglobin, erythrocytes,² and

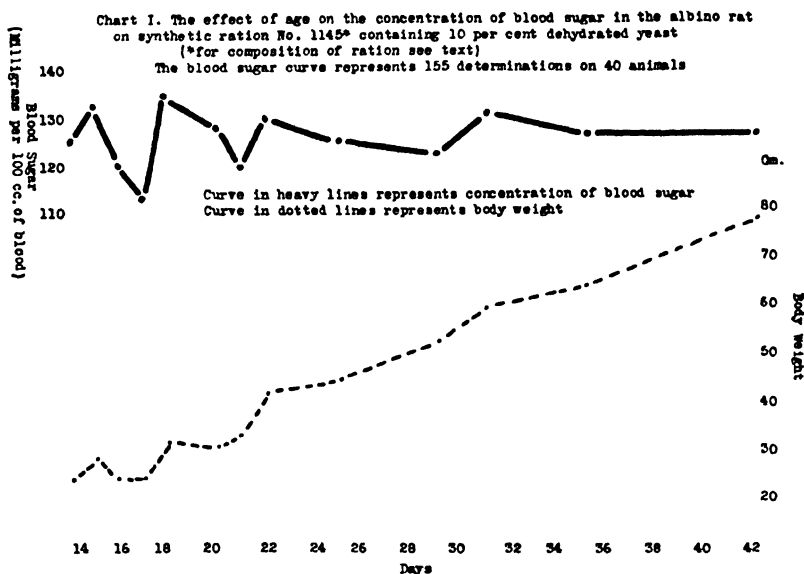


CHART I.

serum proteins. All animals were bled peripherally. After a number of preliminary trials we found that there were less variations in determinations of blood constituents, since as little as 0.05 cc. of blood had to be employed for micro determinations, if we used non-oxalated blood. For the determination of all the above mentioned blood constituents we used 0.31 cc. of blood. The Kramer-Gittelman micro method for blood sugar (4) was found most suitable for our purpose, since only 0.05 cc. of blood

² Credit is due Miss D. J. Walker for making most of the erythrocyte counts.

is necessary, and the determinations checked with the gasometric method of Van Slyke and Hawkins (5) to the extent of ± 1 per cent.³ The rest of the blood constituents were determined according to technique described in the preceding paper (2).

Chart I shows the effect of age on the concentration of blood sugar on synthetic Ration 1145, abundant in vitamins B and F. It will be noted that between the 14th and 22nd day of lactation the concentration of blood sugar was between 113 and 135 mg. per cent. Between the 22nd and 42nd day, representing a post weaning period, the blood sugar maintained almost a plateau, fluctuating from 120 to 130 mg. per cent. On Stock Diet 1 the blood sugar between the 18th and 38th day varied from 110 to 138 mg. per cent. On neither of these two types of diets did the concentration drop below 110 mg. per cent. With these figures as a basis of comparison, all of our pathological animals showed marked hypoglycemia during the vitamin B depletion period long before any losses of body weight had taken place.

From the standpoint of concentration of blood sugar, the pathological animals fall into two groups, one that showed progressive hypoglycemia, and the other which, during the early period of vitamin B depletion, had a hypoglycemia of 10 to 20 per cent, followed by a precipitous drop of 40 to 50 per cent during the pre-mortal state. All the nurslings suffering from a deficiency of the vitamin B complex showed the progressive type of hypoglycemia, while only seventeen young out of forty-four young in the prolonged maintenance state of uncomplicated vitamin B deficiency manifested that state of hypoglycemia.

Space does not permit us to show all of our results in detail. We are, therefore, submitting two illustrations, in Charts II and III, which are representative of the two types of hypoglycemia encountered. In one case we are also showing the effect of vitamin B therapy on growth, concentration of blood sugar, hemoglobin, erythrocytes, and serum proteins. Seven additional vitamin therapy cases were studied.

Chart II. Nursling S-195.—The progressive development of accentuated hypoglycemia is very striking in this nursling. The blood sugar was reduced in 10 days from 103 to 55 mg. per cent.

³ We used a micro method, kindly furnished us by Dr. Van Slyke.

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The anemia and anhydremia are also quite evident in this animal. The anemia was evidenced not only by the precipitous drop in hemoglobin, during the last 4 days of the experiment, but also in

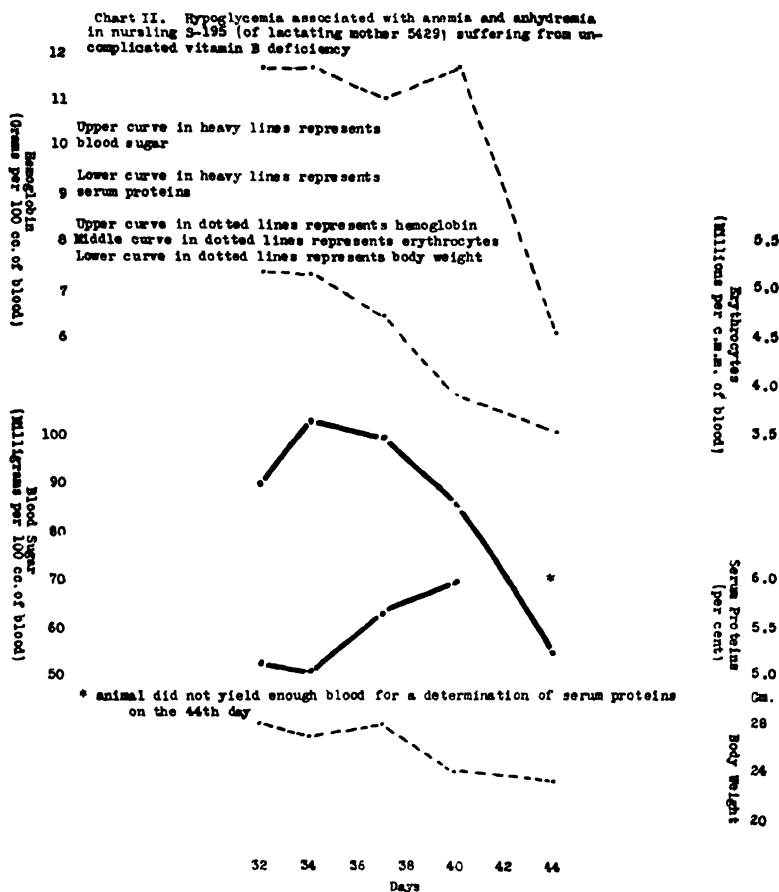


CHART II.

the continuous reduction in the concentration of the erythrocytes. Between the 32nd and 40th day there was an increase of 20 per cent in the concentration of serum proteins, although there was a loss of body weight to the extent of 14 per cent during the same interval, i.e. anhydremia.

Chart III. Nursling S-185.—The biochemical changes in this nursling preceding vitamin B therapy are of special interest. During the period the hemoglobin was falling (35th to 45th day) the

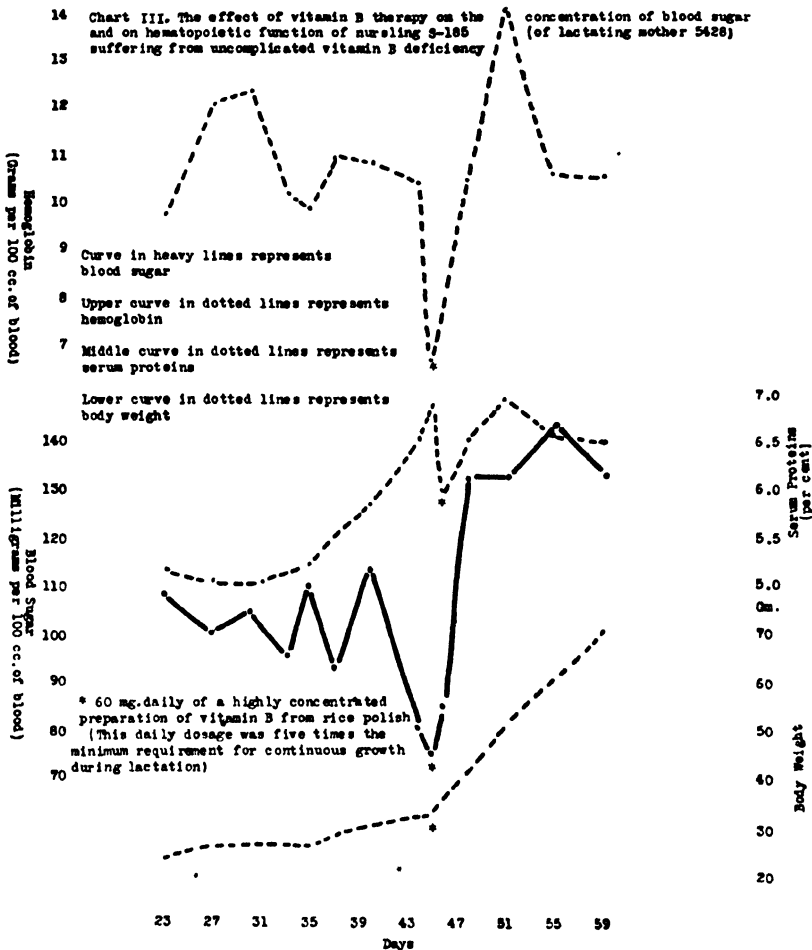


CHART III.

concentration of serum proteins was appreciably increasing. Such findings we interpreted to signify that there might be water lost from the blood serum and at the same time an anemia produced

in the individual. We have observed in a number of control animals that the concentration of serum proteins and hemoglobin does not necessarily occur in definite proportions. For instance, we found in several animals on control Ration 1145 the concentration of serum proteins to be 4.5 per cent and the hemoglobin concentration in the same individuals that were making excellent growth to vary from 7 to 12 gm. per 100 cc. of blood.

At the point of the precipitous drop of blood sugar and hemoglobin this nursling showed advancing symptoms of polyneuritis, and vitamin therapy was then instituted. The daily administration of 60 mg. of a highly concentrated extract of vitamin B from rice polishings⁴ was initiated. The effect of vitamin B therapy was most pronounced on the concentration of blood sugar. In 3 days there was an increase of 77 per cent and the higher concentration was maintained throughout the rest of the experimental period. The influence of vitamin B administration on the regeneration of hemoglobin was equally marked. During the vitamin B depletion period the erythrocytes fell from 5.12 to 4.03 millions per c.mm. and on the 59th day when the experiment was terminated the concentration of red blood corpuscles was 6.80 millions per c.mm. (not shown on chart). The effect of vitamin B therapy on growth was also quite pronounced.

DISCUSSION.

While the character of the hematopoietic disturbance was different in each of the nursing young suffering from uncomplicated vitamin B deficiency, as evidenced by concentration of hemoglobin, erythrocytes, and serum proteins, the pathological symptom common to all of such nurslings was hypoglycemia. That the hypoglycemia was frequently progressive, and not just a premortal state, was apparent in seventeen cases out of forty-four studied. Since the hypoglycemia was quite evident during a period of prolonged maintenance produced by vitamin B depletion, so that the condition was not complicated by appreciable changes in body weight, there can be no question, then, that vitamin B had a determining influence on carbohydrate metabolism. Our results

⁴ Prepared by one of us (B. S.) by a chemical procedure to be described later.

of hypoglycemia in polyneuritic nursing young of the albino rat are in agreement with the findings of Suzuki (6) of hypoglycemia in infantile beriberi. The hyperglycemia reported in avian beriberi (7) and in full grown rats suffering from a deficiency of the vitamin B complex (8) are not comparable with our work on nursing baby rats, and no attempt has, therefore, been made to review critically that literature. We are continuing our studies on animals during the post weaning period and also on adult rats.

The frequent rapid increases in the concentration of serum proteins encountered during periods of prolonged maintenance, and particularly during intervals of loss of body weight, unquestionably establish the fact that in uncomplicated vitamin B deficiency of nursing young of the albino rat we have pronounced anhydremia.

The marked anemia observed, as evidenced by the reduction in the concentration of hemoglobin to the extent of 100 per cent, may be open to criticism. In order to secure enough blood for the determinations of all the constituents studied, we were obliged to draw as much as 0.31 cc. of blood from baby rats, some weighing as little as 20 gm. If we calculated the blood volume as 6 per cent (9) (in the absence of direct blood volume determinations) we had to remove, in some cases, 26 per cent of the total blood of the animal at each bleeding. Realizing this situation, we planned to bleed the nurslings not more than twice a week. On the other hand, the external symptoms frequently warranted more frequent bleedings, in order to secure additional information before the onset of the premortal state of the animal. But, granting that the anemia encountered in our pathological nurslings had been produced partly by removal of blood from the animals, these young must have been unable to regenerate their blood because of vitamin B deficiency, since we bled the nurslings that received vitamin B therapy just as frequently, and the result was rapid blood regeneration instead of a development of anemia.

A word of explanation is necessary as to the vitamin B dosage employed in eight therapy experiments. A biological assay of the concentrated preparation of vitamin B from rice polishings employed in this investigation on several hundred individuals by our quantitative biological method (10) revealed a potency of 1 gm. containing 80 vitamin B units (uncomplicated); i.e., 12 mg.

were found sufficient to produce a growth of 10 gm. in a 30 gm. nursing young during a period of 7 to 10 days. In an attempt to save nursing young in the terminal stages of uncomplicated vitamin B deficiency even 3 times the 12 mg. dosage proved ineffectual. We, therefore, used 60 mg., or 5 times the minimum dosage with great success. To date we have succeeded in saving three individuals in the terminal stage of the avitaminosis (two of which were in marked opisthotonos) that were failing, gasping for breath, by proceeding with administrations of 60 mg. daily allowances of the concentrated extract of vitamin B from rice polishings.

Our food consumption records show that the lactating mothers, whose young developed the avitaminosis, consumed daily about 50 per cent less metabolizable food products than is necessary for the optimum development of nursing young during lactation. Our tentative hypothesis, then, is that the insufficient food intake for normal lactation results in less carbohydrates accessible to the nursing young for energy metabolism, and hence the hypoglycemia.

The biochemical changes in the blood of the nurslings that received vitamin B therapy might be attributed to growth produced by increase in food consumption, which in turn, was influenced by the physiological action of vitamin B. The increases in the concentration of blood sugar and the regeneration of hemoglobin following vitamin B therapy, however, proceeded at a much more rapid rate than the increase of body weight.

SUMMARY.

1. Nursing young of the albino rat suffering from uncomplicated vitamin B deficiency develop marked hypoglycemia at a stage of the avitaminosis before loss of body weight has occurred.

2. In such avitaminosis nursing young of the albino rat also develop anhydremia and marked disturbance in hematopoietic function.

3. Vitamin B therapy produces a rapid increase in concentration of blood sugar, and a regeneration of blood in polyneuritic nursing young of the albino rat.

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THE SOLUBILITY OF GASES IN BLOOD AND BLOOD FLUIDS.

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In connection with the problem of the determination of the cardiac output by the use of ethylene (13), it seemed of interest to investigate further the nature of the forces which make the solubility of this gas $1\frac{1}{2}$ times as great in human blood as it is in water. The general problem of the nature of the factors which determine the solubility of gases in blood is of importance not only because of the theoretical considerations involved but also because of the practical application of these values to such problems as the determination of the cardiac output, the general problem of anesthesia, and the like. Despite the number of determinations of the solubility of gases in blood which have been made in the past, there is still much confusion regarding the factors which determine the solubility of a given gas in blood or blood fluids. Of particular practical interest, too, is the problem of the variation of this solubility with different bloods of the same and different species. The present communication reports data on the solubility of several gases in water, blood, and aqueous solutions of certain blood constituents for the value which these data may have on the further elucidation of the problems mentioned above.

Experimental Methods.

Owing to the diversity of the gases and solvents used in the present study, the methods employed had to be varied from case to case. The data of Table I were obtained by the use of the method employed by Geffcken (8) as modified by Findlay and his co-workers (6) in their studies on solubility. This method, which is a modification of the original method of Ostwald (14), measures the actual volume of a gas which is absorbed by a

liquid and permits making a series of successive determinations at increasing pressures. In this way one can study the effect of variation of pressure on the solubility of a gas. Despite this advantage of the method, it proved untrustworthy at very low or high pressures. The method proved particularly inaccurate in the case of measurements of the solubility of nitrous oxide and hence the results obtained with this gas are not recorded in this paper. This has also been the experience of Manchot (12) who, too, has criticized the method of Geffcken (8).

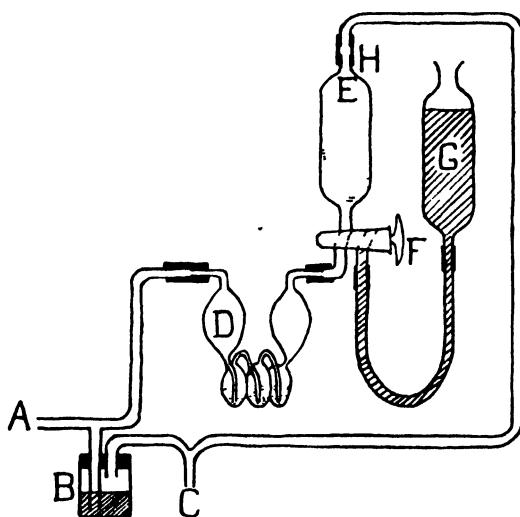


FIG. 1. Apparatus for saturation by the bubbling method.

Saturation Methods Employed.

For the determinations at 37.5° the “bubbling” and “tonometric” methods as used by Van Slyke and his collaborators were employed (21). For protein-free solutions (water and blood fat) the bubbling method was used, while the tonometric method of Austin *et al.* (2) was employed for blood, serum, or corpuscles. The gases employed were led from commercial tanks, through a purifying train and into the saturator.

A convenient arrangement for saturation by the bubbling method is represented in Fig. 1. The purified gas enters at A.

B is a mercury trap through which the gas may escape at *C* which leads out of a window. In this way explosive or noxious gases are prevented from escaping into the room. *D* is a Geissler bulb by which the incoming gas is saturated with water vapor at the temperature of the experiment. The gas then passes through the vessel *E*, which contains the solvent, and escapes at *C*. *D* and *E* are submerged in a constant temperature bath. When equilibrium has been attained the cock *F* is turned through an angle of 180° (without removing *D* and *E* from the bath) and mercury from the receiver *G* is allowed to enter *E*, displacing the gas and allowing a little of the solution to rise through the tube *H*. A clamp is then applied at *H* and the apparatus removed from the bath. The transfer of the solution from *E* to the evacuation chamber was carried out without contact of the solution with the air, according to the procedure described by Van Slyke and Neill (19).

All determinations were made in a constant temperature bath maintained within 0.05° as recorded by a thermometer calibrated against one corrected by the Bureau of Standards.

Analytical Methods.

Analyses of nitrogen were made directly in the solutions by the manometric gas apparatus of Van Slyke and Neill (19). The special pipette and technique for transfer of the solution, as described by these workers, were employed. In the case of the other gases studied, the solution was transferred in a similar manner to the apparatus of Van Slyke (18) and the gas removed by three evacuations. The evacuated gas was transferred to a sampling tube and then analyzed. Ethylene analyses were carried out as described in a previous paper (13). Acetylene was analyzed by absorption in alkaline mercuric cyanide in the apparatus, supplied with a 25 cc. burette, described elsewhere (10).

The water contents of the solutions were determined by drying 5.00 or 10.00 cc. samples overnight at 110° and determining the loss of weight. Fat determinations were made by the Rösse-Gottlieb method as adopted by the Association of Official Agricultural Chemists.

Source of Materials.

The rabbit and dog blood used in this work was obtained by the use of a local anesthetic, as described in a previous paper (9). Heparin was used as an anticoagulant in all cases. Plasma was obtained from this blood by centrifugalization. The hemoglobin solutions used were prepared according to the procedure of Adair (1).

TABLE I.

Solubility of Ethylene in Water, Dog Plasma, Dog Whole Blood, and Dog Hemoglobin Solution at 25.00°.

The solubilities are expressed in terms of the Ostwald coefficient. The hemoglobin solution contained 8.5 gm. of purified hemoglobin in 100 cc. of solution. The values given are interpolated to correspond to exact pressures obtained from a *pressure-solubility chart of the experimental data.*

Partial pressure of ethylene.	Solubility in:			
	Water.	Plasma.	Hemoglobin	Whole blood.
550	0.112	0.114	0.104	0.141
600	0.113	0.114	0.104	0.141
650	0.113	0.114	0.105	0.141
700	0.112	0.115	0.106	0.142
750	0.113	0.115	0.105	0.142
800	0.113	0.115	0.106	0.141
850	0.113	0.116	0.106	0.143
900	0.114	0.115	0.106	0.142
950	0.113	0.116	0.107	0.143
1000	0.113	0.117	0.107	0.144

The blood lipoids¹ were prepared by extracting blood corpuscles with ethyl ether and petroleum ether as in the Rösé-Gottlieb method for the determination of total fat.

The samples of normal human blood were obtained by venepuncture; heparin was used as an anticoagulant. The pathological

¹ The term lipid is used throughout this paper to denote those blood constituents which are removed by petroleum ether and ethyl ether. These substances represent probably a heterogeneous mixture, for which a more exact name is not available. The substances not removed by the above reagents are the salts, proteins, hemoglobin, and probably some part of the corpuscular stroma.

specimens were obtained from ward cases through the courtesy of Dr. G. A. Harrop, Jr.²

Results.

The results of a series of determinations at varying pressures of the solubility of ethylene in water, plasma of dog blood, and an aqueous solution of hemoglobin prepared from the same dog blood are given in Table I. It was the purpose of these experiments to determine if ethylene was combined with hemoglobin and if the latter was involved in the increased solubility of ethylene in blood as compared to water.

TABLE II.

Solubility of Several Gases in Water and in Aqueous Lipoidal Suspension Containing 0.3 Gm. of Blood Lipoid in 100 Cc. of Water.

The solubilities are expressed in terms of the Bunsen coefficient and were determined at 37.5°.

Gas.	Solubility in water.	Solubility in lipoidal suspension.
Nitrogen	0.0128	0 0133
Ethylene.....	0 078	0 120
Acetylene.....	0 747	0 748

The results of Table I show that ethylene in water, plasma, or hemoglobin solutions follows Henry's law. The slight inconstancy of the results is attributed to experimental error. This adherence to Henry's law has been found to be the case in many previous studies of the solubility of gases in blood. Opposed to this view, however, are the results of Findlay and Creighton (5) who observed marked deviation from Henry's law in the case of nitrous oxide and nitrogen dissolved in blood and other colloidal solutions. Conant and Scott (4) have more recently reported similar observations in the case of nitrogen in hemoglobin solutions. The adsorption of the relatively inert nitrogen or nitrous oxide by hemoglobin in aqueous solution is rather unexpected and not reconcilable with the behavior of hemoglobin towards much more active substances.

² The author is also indebted to Dr. Alan M. Chesney for a sample of rabbit blood.

Solubility of Gases in Blood Lipoids.

Since neither the plasma nor the hemoglobin of dog blood contributes appreciably to the increased solubility of ethylene in the whole blood (Table I), it was concluded that this increased solubility was due to the presence of lipoids. This influence of the lipoidal constituents of the blood cell in increasing the solubility of gases in blood has been emphasized by a number of previous workers (15, 17, 20, 21, 23). Only indirect experimental proof of this fact has, however, been adduced. Comparative studies of the solubility of gases in other lipoidal solvents such as olive oil or animal fat (22, 23) have been made, but such comparisons of the relative solubilities of a gas in different solvents are, however, rather inadequate as is shown in Table I of the paper by Van Slyke *et al.* (20).

In order to determine more accurately the effect of the lipoidal constituents of the blood on the solubility of gases, the lipid was extracted, as described above, and the solubilities of several gases in an aqueous suspension of this lipoidal material were determined. The results are shown in Table II which also gives the solubilities (Bunsen coefficient) of the gases in water as determined in parallel experiments under the same conditions. The data of Table IV clearly show the increase in solubility which the presence of the lipoids exerts.

It is, of course, evident that the extracted lipoids may not necessarily exert the same solvent action under the conditions of these experiments as they do when present in their native condition in the blood. However, the results indicate a relative agreement between the general behavior of the solubilities of the gases in blood (their solubility in lipid being assumed the same as found in Table II) and the experimentally observed values.

Solubilities of Acetylene and Ethylene in Various Blood Fluids.

In Tables III and IV is collected a series of determinations of the solubilities of acetylene and ethylene in various fluids. Ethylene shows much more marked variations in its solubility than does acetylene. Despite the fact that both gases are inert as compared to chemically active gases such as carbon dioxide, there are wide variations of the solubilities of these gases as compared to their

solubilities in water. This fact is significant and demonstrates the invalidity of the simple considerations advanced originally by Bohr (3) to explain the solubility of gases in blood.

TABLE III.
Solubility of Ethylene in Water, Blood, and Blood Fluids at 37.5°.

Solvent.	Solubilities.		Relative solubilities.	
	α	α°	$\frac{\alpha \text{ solution}}{\alpha \text{ water}}$	$\frac{\alpha^\circ \text{ solution}}{\alpha \text{ water}}$
	<i>cc. C₂H₄ per cc. solution</i>	<i>cc. C₂H₄ per gm. H₂O</i>		
Distilled water.....	0.078	0.0785		
Whole human blood.....	0.123	0.156	1.58	2.00
“ dog “	0.141	0.167	1.81	2.14
“ rabbit “	0.128	0.148	1.64	1.90

TABLE IV.
Solubility of Acetylene in Water, Blood, and Blood Fluids at 37.5°.

Solvent.	Solubilities.		Relative solubilities.	
	α	α°	$\frac{\alpha \text{ solution}}{\alpha \text{ water}}$	$\frac{\alpha^\circ \text{ solution}}{\alpha \text{ water}}$
	<i>cc. C₂H₂ per cc. solution</i>	<i>cc. C₂H₂ per gm. H₂O</i>		
Distilled water.....	0.747	0.752		
Whole dog blood.....	0.759	0.943	1.02	1.25
Dog plasma.....	0.690	0.751	0.92	1.00
“ corpuscles.....	0.778	0.986	1.04	1.31
Whole human blood.....	0.740	0.916	0.99	1.22
“ rabbit “	0.703	0.812	0.94	1.08
“ blood in case of polycythemia vera.....	0.710		0.95	
Whole blood in case of myeloid leukemia (anemia and relative lipemia).....	0.735	0.881	0.98	1.17

The lipoidal constituent of the red blood cell seems to be the factor which brings about this effect. In the case of the relatively *lipoid-insoluble* gases, oxygen, carbon dioxide, nitrogen, or acetylene, the effect is comparatively small, although still significant. In the case of ethylene, a gas much more soluble in lipoids, the effect is very great.

It must be remembered that in dealing with bloods of various origin, one must consider not only the varying lipid content (both qualitatively and quantitatively, but also the varying water content of the blood in question. Thus the conclusion of Lehmann (11) that neither the hemoglobin nor the lipid content determines the solubility of ethyl iodide in blood seems unjustified in view of his failure to take into account the variations in both of these factors. The low solubility of acetylene (observed also by Schoen and Sliwka (16)) or of ethylene in rabbit blood as compared with dog blood is thus accounted for by its low lipid content as compared to the other bloods studied. The diminution in solubility of acetylene in the blood of a case of polycythemia vera (erythrocyte count 6,800,000) is accounted for by the preponderatingly greater influence of the diminution in the water content of the blood over the increase in its lipid content. In the case of myeloid leucemia studied, there was an anemia (erythrocyte count 2,200,000) and a relative lipemia (total fat content 0.864 per cent), the final effect being such as to give a solubility but little different from normal blood.

The effects outlined above far overbalance the possible effects of the blood colloids on the solubility of gases. That colloids do affect the solubility of gases has been claimed by Findlay and his coworkers (5, 6) and by Gatterer (7). It seems most likely, however, that this effect is rather small in the case of chemically inert gases such as acetylene or ethylene.³

The practical application of these findings to the problem of the determination of the cardiac output in man, by the use of foreign gases, has already been discussed (10).

SUMMARY.

The solubilities of ethylene at varying pressures in water, dog blood plasma, and dog hemoglobin solutions were determined at 25.0° and found to follow Henry's law. The solubilities of nitrogen, acetylene, and ethylene were also determined in water and

³ The actual combination, however, of carbon dioxide and hemoglobin has been described by Kato, S., (*J. Biochem.*, Japan, **8**, 167, 187 (1927)) and Henriques, O. M., (*Biochem. Z.*, **200**, 1 (1928)).

compared to their solubilities in a suspension of the blood lipoids, at 37.5°. The blood lipoids are shown to increase the solubilities of these gases in aqueous solutions. The solubilities of ethylene and acetylene in human, dog, and rabbit blood and in certain blood fluids were also determined.

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THE LIPID DISTRIBUTION IN NORMAL AND ABNORMAL LIVER TISSUES.

III. THE EFFECT OF DISEASE UPON THE LIPID DISTRIBUTION IN HUMAN LIVER TISSUE.

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In Papers I and II of this series, the lipid distribution in beef and rabbit liver tissue was discussed. It was shown that in normal liver tissue, of the total lipid material present in the tissue, there exists a certain definite relation between the phospholipid and the neutral fat. We have found that in perfectly normal tissue this ratio of phospholipid to neutral fat is constant. MacLean and Williams (1) in 1909 showed that the greater portion of fatty material extracted from liver tissue was present in the phospholipid form. These workers infer that the percentage of phospholipid with relation to the total lipid content may be 84 per cent. In our work, with livers of different species of animals, we have never been able to obtain much better than 75 per cent phospholipid. Mayer and Schaeffer (2) have examined various tissues and found the proportion of lipids in most tissues comparatively constant and even unchanged after fasting. These workers point out that it appears probable that the character of an organ may be conditioned as much by proportion of its constituents as by the specific properties of the constituents themselves. MacLean (3) writes as follows: "The constant presence of the lipins in every cell and their retention even under conditions of extreme emaciation makes it certain that they are essential for maintaining the vital processes of the cell."

Our work to date well supports the contentions of MacLean. We have found that normal liver tissue has a rather constant

lipid content, which for animals of the same species compares favorably within the experimental error. Under abnormal conditions this constancy usually maintains itself. However, there are other facts to be considered as well as the so called lipid constancy of the tissue. Not only is the total lipid material of the tissue, in the main, constant but the relation of the separate constituents to the total lipid material is also constant.

In the general lipid classification as outlined by Bloor (4), we find the lipids divided into three main groups, simple, compound, and derived lipids. For our purpose, it would seem necessary to discuss only the simple and compound lipid material; neutral fat is a representative of the former and phospholipid of the latter. It is possible to express the relation of the phospholipids to the neutral fat in an equilibrium equation: phospholipid \rightleftharpoons neutral fat. In normal liver tissues, this relation is apparently a constant. This ratio has been worked out for beef and rabbit liver material (5, 6) under both normal and abnormal conditions and may be expressed for the normal tissue as follows:

Phospholipid \rightleftharpoons neutral fat		
	per cent	per cent
Beef liver.....	55	45
Rabbit liver.....	55-65	35-45

When, however, certain degenerative changes set in, or abnormal conditions arise, there may be either a change in total lipid content or more generally a shift to the right of the phospholipid-neutral fat ratio. Such an abnormal state as may occur in fatty degeneration may cause this ratio to change from 65:35 to 3.5:96.5 (5, 6). Injection of insulin into perfectly normal test rabbits, caused a marked change in the phospholipid-neutral fat ratio. The following tabulation shows the effect of various abnormal conditions upon the phospholipid-neutral fat ratio of rabbit liver tissue.

Normal.....	65:35
Insulin-treated.....	4:96
Phosphorus-treated.*.....	4:96
Castrated†.....	26:74

* Rabbits were given phosphorus in olive oil; animals killed in 36 hours; livers removed and examined.

† Series of males castrated and killed 24 hours later; livers removed and examined.

In the animals given phosphorus, we know that severe fatty degeneration of the liver tissue occurred and this is in accordance with our suggested theory that any condition which will tend to destroy the normalcy of the organ or tissue will cause the phospholipid-neutral fat ratio to change. In a former paper (6) we have pointed out two possible effects of insulin upon the animal economy: first, over a long period of time, insulin causes a decrease in the total lipid content of the liver; and secondly, over a short period, insulin causes a marked change in the phospholipid-neutral fat ratio.

Since it was found that abnormal conditions caused such great differences in the phospholipid-neutral fat ratio in beef and rabbit liver tissue, it seemed of interest to ascertain if such changes of ratio occurred with human liver tissue. It was our purpose to determine just what changes had taken place in the phospholipid-fat ratio of the human liver under the influence of disease. For working material we were dependent upon such cases as came to hand in the surrounding hospitals. In the work to follow, we have classed as normal liver tissue only such organs as appeared after postmortem operation to be perfectly healthy and free from all signs of degeneration of any kind. For diseased tissue, we used those that came to hand, paying particular attention to the organs which showed distinct signs of degeneration.

EXPERIMENTAL.

The liver tissue was removed as soon after death as practical and immediately cut into very small pieces. This material was then dehydrated with absolute alcohol at a temperature of 35°. After preliminary dehydration, the total lipid material was extracted from the tissue with boiling absolute alcohol. After extraction, the alcoholic extract was concentrated *in vacuo* at a low temperature. The extract remaining after concentration was treated with ether and the lipid material removed. The ether solution was allowed to stand for several hours in order that any water might separate. The ether solution was then made up to 500 ml. with anhydrous ether. An aliquot portion of the ether solution was used for the determination of total lipid material. The remaining solution was used for the separation of the following fractions: (1) total phospholipids, (2) solid and liquid fatty material. Each of these fractions was then subjected to the

following analysis: percentage of mixed fatty acids; percentage of solid and liquid fatty acids; percentage of brominated acids

TABLE I.
Lipid Distribution in Human Liver Tissues.

Sample No.....	1*	2	3	4	5	6	7	8
Total lipid extracted.....	3.45	3.04	2.88	2.00	3.94	5.41		5.16
Phospholipid.....	49.80	58.90	38.80	44.50	16.60	31.10	39.93	33.20
Solid fatty material.....	2.97	3.39	5.94	6.20	1.00	2.30	9.92	12.80
Liquid " "	48.00	40.50	53.60	51.20	82.40	66.60	57.13	54.00

* The sample numbers refer to samples cited in the text.

TABLE II.
Fatty Acid Distribution of Lipids in Liver Tissue.

	Sample 1*.	Sample 3.	Sample 4.	Sample 6.	Sample 7.
Phospholipid fraction.					
Iodine No. of total lipid.....	85.6	73.7	71.3	84.2	94.1
" " " mixed fatty acids.....	121.0	101.0	105.0	114.0	103.0
" " " liquid " "	199.0	187.5	151.0	185.0	148.0
Per cent of mixed fatty acids.....	63.4	60.0	51.0	68.0	68.0
Solid fatty acids, per cent of mixed fatty acids.....	34.4	41.0	31.3	40.4	26.0
Liquid fatty acids, per cent of mixed fatty acids.....	65.6	59.0	68.7	59.6	74.0
4-Bond acids, per cent of liquid fatty acids.....	14.0	14.2	18.3	25.2	12.0
Liquid fatty fraction.					
Iodine No. of total lipid	103.7	103.0	112.0		81.2
" " " mixed fatty acids.....	119.0	115.0	130.4		83.6
" " " liquid " "	176.0	151.0	148.0		153.0
Per cent of mixed fatty acids.....	76.0	80.5	78.0		81.2
Solid fatty acids, per cent of mixed fatty acids.....	29.8	21.0	38.0		34.0
Liquid fatty acids, per cent of mixed fatty acids.....	70.2	79.0	62.0		66.0
4-Bond acids, per cent of liquid fatty acids.....	14.4	11.4	14.4		10.0

* The numbers refer to samples cited previously.

(insoluble in ether) in the liquid fatty acids; iodine values of the fraction, mixed acids, and liquid fatty acids.

The specimens examined are listed as follows:

Sample 1.—Female, age 35 years. The liver appeared healthy; histological examination showed no degeneration. This was taken as a normal specimen.

Sample 2.—Female, age 22 years; general peritonitis. The liver appeared to be in healthy condition, histological examination showed no degeneration.

Sample 3.—Male, age 37 years. The liver showed distinct signs of fatty degeneration; macroscopically it appeared yellow, streaked, and spotted.

Sample 4.—Male, age 35 years; cerebral thrombosis associated with cerebral edema and chronic adhesive pleurisy. The liver showed signs of fatty degeneration.

Sample 5.—Male, age 21 years; bilateral pulmonary tuberculosis with extensive caseation, necrosis, and cavitation. Further examination showed early tuberculous enteritis, peritonitis, and hepatitis. The liver was yellow and spotted and in very poor condition and indicated extensive fatty degeneration.

Sample 6.—Female, age 35 years; chronic peritonitis; hepatitis with toxic degeneration. Histological examination showed the liver to be in poor condition.

Sample 7.—Male, age 30 years; pneumonia. The liver showed distinct signs of fatty degeneration.

Sample 8.—Child, male, died at birth. No nourishment was received and since the liver had not functioned in the normal sense, it cannot be classed as a normal organ.

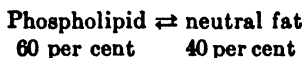
Table I shows the lipid distribution of the various human liver tissues examined.

Table II shows the fatty acid distribution of the lipids obtained from some of the liver tissue examined.

DISCUSSION.

From an examination of Table I, the following conclusions can be drawn.

(a) Samples 1 and 2 represent as nearly normal liver tissue as could be expected from the cases available for analysis. Taking these specimens as representative of a possible normal condition, we can express the equilibrium equation



This ratio is practically identical with the ratio obtained for normal beef and rabbit liver tissue.

(b) Samples 3 and 4 may be said to represent average fatty degeneration conditions, and it is seen that the phospholipid-neutral fat ratio has been shifted markedly to the right, being shown in Sample 3 as 38.8 to 61.2 and in Sample 4 as 44.5 to 55.5. Sample 5 showed histologically severe fatty degeneration and in our examination gave a phospholipid-fat relation of 16.6 to 83.4, which is in line with what should occur if this equilibrium relation is of value.

(c) Sample 7 shows the effect of pneumonia upon the lipid distribution of the liver, and it is seen that the toxic poisons have caused a distinct change in the lipid material of this organ. Sample 8 cannot be taken as representative of any one class and is given only for general interest. This liver can only be taken as embryonic tissue, and it may well be that the lipid distribution in it is representative of the embryonic state.

(d) The total lipid content is of fair constancy in both the normal and abnormal tissue, especially in consideration of the variety of diseased organs. In most of the abnormal cases it is also to be noted that the percentage of solid fatty material is greater than in the normal states.

(e) The chemical examination checked well with the histological examinations conducted in the hospital at the time of the post-mortem operation.

Fatty Acid Distribution.

We find that the fatty acid distribution in human liver tissue is for all practical purposes approximately the same as that for beef and rabbit tissue. The unsaturation of the liquid fatty acids is of the same order as that of other liver material examined to date. The 4-bond acids, which represent arachidonic acid, are distributed throughout both the phospholipid and liquid fatty fractions to about the same degree. Leathes and Meyer-Wedell (7) brought forward evidence that desaturation of the fatty acids takes place in the liver. Later MacLean pointed out that if the fatty acids were built up into phospholipids before desaturation, it would be expected that the highly unsaturated acids would be confined to the phospholipid fraction. Our investigations up to the present time have shown that the unsaturation of the fatty acids is not confined to the phospholipid fraction but that in most

cases the neutral fat fraction contains but little less unsaturated fatty acids. The 4-bond acids are distributed throughout both the phospholipid and the neutral fat fractions.

The iodine values of the fatty acids of the neutral fat fraction are somewhat lower than those of the phospholipid fraction, but sufficiently high to indicate strongly that the unsaturated fatty acids are by no means confined to any one fraction.

As pointed out by Bloor and also in our previous papers, liver lipids apparently contain no 3-bond acids but do contain certain amounts of 2-bond acids. From the percentage of 4-bond acids and the iodine value of the liquid fatty acids, the amount of linoleic or 2-bond acids can be readily calculated. The 2-bond acids in the phospholipid fractions show a value of approximately 70 per cent.

Unsaponifiable Matter.

After saponification of the fraction there was usually a certain amount which was unsaponifiable under the conditions maintained. As a general thing, the amount of the unsaponifiable material was less in the phospholipid fraction than in the neutral fat fraction. In all the cases examined the phospholipid fraction showed an average percentage of unsaponifiable matter of about 5, while the neutral fat fraction showed about 10 per cent. On further examination of this material, it was found that the cholesterol was practically all in the free state.

There are many indications that the lipids play an important rôle in fat metabolism, but just what is that rôle? It has been suggested that both phospholipids and neutral fat are stored in the tissue, but the writer believes only the neutral fat to be stored material and the phospholipids to be active in the actual metabolism of the organ. This contention is supported by all our work. In every case in which the actual functioning of the organ is disturbed, we find the lipid distribution changed to the disadvantage of the phospholipid content.

CONCLUSION.

The lipid distribution and fatty acid distribution of human liver tissue under normal and abnormal conditions have been investigated. The data obtained support the work with beef and rabbit

liver tissue. Pneumonia, tuberculosis, and fatty degeneration alter the phospholipid-fat ratio of the normal tissue, the phospholipids decreasing proportionally with the stage of the disease.

The writer wishes to thank Dr. A. O. Brines of the Detroit Receiving Hospital for his courtesy and kindness in furnishing the material and for his kindly encouragement during the work.

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ACETONE AS A CONTROL SUBSTANCE FOR RESPIRATION AND GAS ANALYSIS APPARATUS.

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Boston.)*

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For many years the standard substance for control tests of respiration apparatus and gas analysis apparatus has been ethyl alcohol. This has served the purpose admirably, but has certain defects as an ideal chemical for general use. Alcohol is hygroscopic, each lot must be standardized, and there are legal restrictions in its purchase which hinder its universal application to chemical uses. It would be ideal also to have an alternate substance to use, when there is doubt as to the purity and concentration of one's supply of ethyl alcohol. For a number of years we have been experimenting with acetone in the testing of respiration apparatus and gas analysis apparatus. Acetone possesses qualities which render its use of advantage. It can be bought in a high degree of purity and therefore does not have to be standardized. It burns very readily in air, is not hygroscopic, and the ratio of carbon dioxide produced to oxygen required in its combustion is 0.75, a quotient within the range of normal respiratory quotients of man. One quality which acetone possesses, however, hindered us for some time in securing satisfactory percentage recoveries of carbon dioxide and oxygen values; that is, it penetrates rubber so readily that the smallest rubber connection in an apparatus for the supply of acetone to the burner prevents 100 per cent recovery of carbon dioxide and oxygen values. This difficulty can be overcome in two ways, either by the use of mercury-sealed joints, or by means of an all glass apparatus. An ordinary burette does not meet the requirements, because the effect of gravity upon the flow of acetone is to slow the rate in the

course of emptying the burette. A burette made on the Mariotte principle is found to serve the purpose. Such a burette has been used with alcohol by Cappellen and Noyons (1925).

Density of Acetone.—As the measurements of acetone burned were always made by means of a burette, it was necessary to convert volume to weight, and for this purpose the density value is better than specific gravity. The density was determined at 20° by means of a 50 cc. Squibb pycnometer with a graduated scale. Two determinations on the acetone used here (Baker and Adamson c.p.) gave 0.7939 and 0.7935.

Coefficient of Expansion of Acetone.—Since the temperature of the acetone may not always be 20°, it is necessary to know the magnitude of the correction due to any change of temperature. The coefficient of expansion from 18° to 22° was determined by means of the 50 cc. Squibb pycnometer and was found to be 0.00142 per degree.

Apparatus for Control Tests of the Gasometer Method with Acetone.—The apparatus used with the gasometer method is shown in Fig. 1. It consisted of a burette, *A*, lamp, *B*, and small spirometer, *C* (Carpenter, 1915), moved by a windshield wiper, *D* (Benedict, 1925). The burette was one of 25 cc. capacity modified into a burette of the Mariotte principle. An ordinary Pyrex burette without stop-cock was taken and into it at the top was fused a uniform glass tube, *K*, of 6.5 mm. outside diameter. The stop-cock, *E*, was then fused onto the small tube and the side opening, *F*, was provided, which could be closed with rubber tubing and a pinch-cock. The burette, thus modified, was then calibrated with distilled water at 20° for the conversion of apparent volumes into true volumes, and with the fusing of the Pyrex capillary tube, *L*, and burner to the lower end of the burette, it was ready for use. When the apparatus was used, it was mounted upon a tripod with an adjustable screw in one of its legs, the rate of flow being regulated by the tilt of the burette. The burner should be at a slightly lower level than the opening of the inner tube of the burette. A piece of asbestos fiber serves as a wick.

Control Tests of Gasometer Method with Acetone.—The results of a series of experiments with the above arrangement and Tissot valves and gasometers are given in Table I. Analyses of the air collected in the gasometers were made in duplicate with the port-

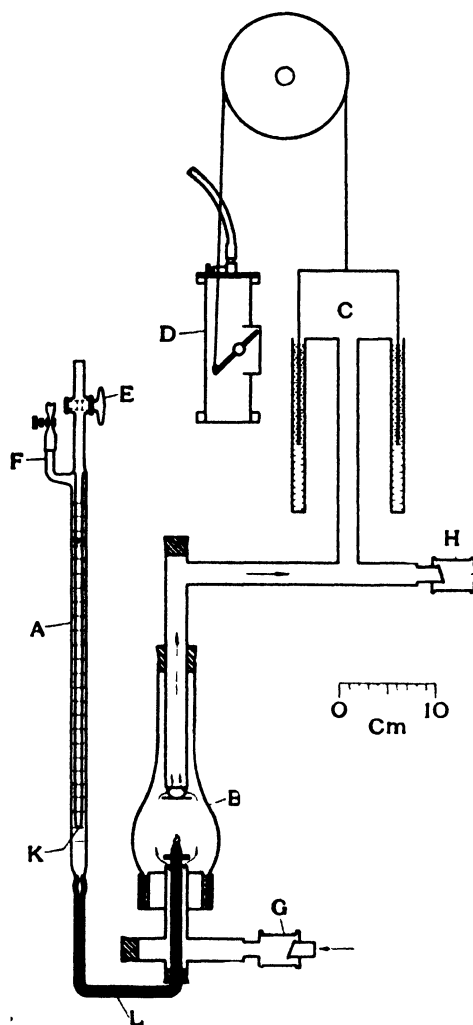


FIG. 1. Arrangement of apparatus for control tests with acetone of the gasometer method of measuring the respiratory exchange. The burette, *A*, was constructed on the Mariotte principle and fused to the capillary burner in the lamp, *B*. The ventilating air current was moved through the apparatus by the small spirometer, *C*, which was raised and lowered by the windshield wiper, *D*. The air entered through the valve, *G*, and left by the valve, *H*. The burette was filled by suction through the side opening, *F*.

able Haldane apparatus, controlled each day by analysis of outdoor air. The periods were approximately 10 to 15 minutes in duration and followed one another in each group without interruption. On November 16, there were two groups. The results of sixteen periods show an average variation of -0.004 from the

TABLE I.
Results of Control Tests with Acetone of the Gasometer Respiratory Exchange Method.

Date.	Acetone burned.	Outcoming air.		Ratio CO ₂ :O ₂ .	Percentage recovery.	
		CO ₂ increase.	O ₂ deficit.		CO ₂	O ₂
1927	cc.	per cent	per cent			
Nov. 14	2.35	3.93	5.27	0.746	100.4	101.0
	1.88	3.47	4.66	0.745	100.2	101.0
	2.35	3.35	4.43	0.756	99.7	98.9
	2.35	4.05	5.42	0.747	100.5	100.8
Nov. 16	2.35	4.19	5.63	0.744	99.5	100.2
	2.35	4.03	5.43	0.742	100.4	101.5
	2.35	4.39	5.92	0.742	98.4	99.4
	2.35	4.01	5.39	0.744	99.2	100.0
	2.35	4.51	6.04	0.747	102.5	102.9
	2.35	4.02	5.38	0.747	99.4	99.8
	2.35	4.78	6.43	0.743	98.2	99.1
	2.35	4.15	5.60	0.741	97.9	99.1
Nov. 18	2.35	3.59	4.86	0.739	102.3	103.9
	2.40	3.96	5.34	0.742	100.7	101.9
	2.35	3.18	4.22	0.754	99.2	98.7
	2.35	3.46	4.62	0.749	99.1	99.2
Average.....				0.746	99.9	100.5

theoretical 0.750 for the ratio, CO₂:O₂, and -0.1 per cent and $+0.5$ per cent variation from the theoretical values of the carbon dioxide produced and oxygen used, respectively, in the combustion of acetone.

Control Tests of Gas Analysis Apparatus with Acetone.—In an earlier publication from this laboratory (Carpenter, 1915, p. 259), it was pointed out that the use of outdoor air as a control for the

accuracy of gas analysis apparatus was not ideal because its composition did not correspond closely to that of expired air, a statement in a measure applicable to that of respiratory chamber air. Control tests by means of burning ethyl alcohol in an air current have been made with an apparatus designed for the analysis of chamber air (Carpenter, 1923). Acetone may be used in the same manner and serves as another check upon the accuracy of the gas

TABLE II.

Carbon Dioxide Increment and Oxygen Deficit in an Air Current from Burning Acetone. (Haldane Portable Gas Analysis Apparatus.)

Date.	Outcoming air.		Ratio CO ₂ :O ₂ .
	CO ₂ increase.	O ₂ deficit.	
<i>1926</i>	<i>per cent</i>	<i>per cent</i>	
Oct. 10	2.02	2.72	0.743
	3.29	4.39	0.749
	3.44	4.62	0.745
	2.32	3.05	0.761
	0.73	0.92	0.793
	10.60	14.13	0.750
Oct. 12	6.26	8.35	0.750
	10.07	13.55	0.743
	9.87	13.02	0.758
Oct. 13	5.68	7.58	0.749
	0.67	0.83	0.807
<i>1928</i>			
Oct. 12	2.82	3.73	0.756
	3.06	4.04	0.757
	2.95	3.95	0.747
	2.86	3.77	0.759

analysis apparatus. In Table II are results obtained with the Haldane portable apparatus in the analysis of air coming from a lamp, or small chamber, in which acetone was burning. The ratio of CO₂ to O₂ found, compared with the theoretical 0.750, was satisfactory in most instances unless the changes in the composition of the air were under 1 per cent. The surprising values are those which show that acetone will burn in air so readily that two-thirds of the oxygen brought in the air current to the lamp may be utilized without seriously interfering with the completeness of combustion.

A control of the accuracy of the larger apparatus (Carpenter, 1923) for the analysis of chamber air was also made with acetone at different times with the results shown in Table III. Again acetone proved to be a suitable substance for the purpose.

Control Experiments with Combinations of Ethyl Alcohol and Acetone.—The ideal control test of a respiration apparatus would be the one that imitates most closely the processes which take place when a biological unit is the subject of the experiment. The ordinary experiment with ethyl alcohol alone has usually

TABLE III.

Carbon Dioxide Increment and Oxygen Deficit in an Air Current from Burning Acetone. (Haldane-Carpenter Gas Analysis Apparatus.)

Date.	Outcoming air.		Ratio CO ₂ :O ₂ .
	CO ₂ increase.	O ₂ deficit.	
<i>1922</i>	<i>per cent</i>	<i>per cent</i>	
July 3	1.506	2.035	0.740
	1.431	1.945	0.736
July 20	0.309	0.407	0.759
	0.297	0.401	0.741
	0.298	0.403	0.739
Oct. 23	0.963	1.303	0.739
	0.941	1.265	0.744
	1.401	1.880	0.745
	1.060	1.408	0.753
<i>1928</i>			
Apr. 26	0.689	0.904	0.762
	0.698	0.931	0.750
	0.753	1.006	0.749

been one in which the combustion was practically uniform from hour to hour, or slightly decreasing. In the biological processes there is practically no constancy, and therefore the most rigid theoretical test of a respiration apparatus would be one in which there were variations both quantitatively and qualitatively. The use of alcohol and acetone in various combinations meets these requirements. Table IV gives the results of four experiments with the universal apparatus (Benedict, 1912, 1924) in which alcohol and acetone in varying amounts were used.

The arrangement of apparatus for the combustion of these

substances was different from any used in the other experiments in this paper. Two 10 cc. burettes graduated to 0.02 cc. with glass stop-cocks were mounted upon a high standard and to the outlets were fused pieces of capillary tubing about 0.5 meter in length. Stop-cocks were then fused to the lower ends of the

TABLE IV.

Control Experiments with Benedict Universal Apparatus with Combinations of Ethyl Alcohol and Acetone.

Date.	Duration.		Amount burned.		Ratio CO ₂ :O ₂ .	
			Alcohol.	Acetone.	Found.	Theory.
1923	min.	sec.	cc.	cc.		
May 1	17	30	1.50	1.39	0.710	0.709
	17	5	1.72	1.53	0.698	0.708
	18	5	0.88	1.77	0.731	0.725
	19	18	3.69		0.668	0.667
May 3	23	8	4.16		0.664	0.667
	18	6	2.08	1.66	0.695	0.706
	20	7	0.92	2.43	0.723	0.729
	18	26	2.22	1.42	0.700	0.702
	14	5		3.17	0.737	0.750
May 4	19	25	2.43	1.74	0.701	0.704
	17	45	0.93	2.70	0.722	0.731
	19	54	2.34	1.66	0.700	0.704
	19	44	1.34	2.51	0.721	0.723
May 5	23	49	1.17	2.79	0.705	0.728
			2.91	0.54	0.680	0.681
	21	37	1.19	2.45	0.720	0.725
	17	19	1.72	0.83	0.687	0.696
	22	22	1.61	1.71	0.711	0.712
Average.....					0.704	0.709

tubing and from these capillary tubing formed a Y which was connected to a burner for the lamp (Carpenter and Fox, 1923). A minimum amount of rubber tubing was used in making the single connection between the burner and the burette combination. The two lower stop-cocks were used for regulating the rate of flow and the two stop-cocks on the burettes for turning on and

off the supply of acetone or alcohol. The burettes were purposely placed as high as possible and the capillary tubing was used in order to overcome the difference in gravity effect at the different levels of the liquids in the burettes. The arrangement lacked the perfect prevention of leakage that we now know is necessary, but the losses were small and did not affect seriously the combined quotient. This is illustrated by a theoretical calculation of the ratio, $\text{CO}_2:\text{O}_2$, for half and half acetone and alcohol, compared with the ratio for the same mixture after 5 per cent loss in acetone. The former gives 0.709 and the latter 0.708.

The agreement of the ratios of CO_2 to O_2 in Table IV is satisfactory. Although the percentage recovery was not as close to theory as we would desire, we have every reason to believe that with a combination arrangement in which all joints were either mercury-sealed or of glass such as was used in the experiments in Table I, percentages of recovery would be obtained comparable to those with the gasometer method. We have not had opportunity since the experiments of 1927 to try this improved combination. Table IV illustrates the possibilities for testing respiration apparatus under conditions in which respiratory quotients vary from one period to another.

SUMMARY.

The use of acetone as a substance for control tests of the gasometer method, and of the Haldane portable and the Haldane-Carpenter gas analysis apparatus is described, and also the use of combinations of alcohol and acetone with the Benedict universal apparatus.

The average of sixteen periods with the gasometer method was 0.746 for the ratio of CO_2 to O_2 and 99.9 per cent and 100.5 per cent for the recovery of the theoretical carbon dioxide and oxygen values.

The average ratio of CO_2 to O_2 with the Haldane portable gas analysis apparatus was 0.751 when the changes in the composition of the air current were 2 per cent or over. The average ratio with the Haldane-Carpenter gas analysis apparatus was 0.746.

The average ratio of CO_2 to O_2 found for the various mixtures of ethyl alcohol and acetone was 0.704 with the Benedict universal apparatus compared with a theoretical average of 0.709.

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A NEW DEFICIENCY DISEASE PRODUCED BY THE RIGID EXCLUSION OF FAT FROM THE DIET.*

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The present paper is the first of a series which the authors expect to offer on the more general subject of the effects of fat in the diet. This paper will be limited to the following topics: experimental methods; description of the new disease; comparative growth and ovulation with and without fat; curing the disease; discussion, and summary.

It is believed that the data presented here definitely settle the uncertainty as to the necessity for fats in the diet (of the rat), and prove not only that ingested fats have a beneficial effect upon the animal but that under the experimental conditions outlined in this paper they are *essential* constituents of the diet. When dietary fats are reduced below a certain minimum, the rat develops a characteristic disease and dies at an early age.

Experimental Methods.

The experimental colony is kept in a spacious north room in which the upper half of the wall is glass. The light conditions are the most constant possible since the north sky furnishes all the illumination throughout the year. The room is maintained at a constant temperature of $24.5^{\circ} \pm 1^{\circ}$.

Individual circular cages, 8 inches in diameter by 6 inches high, made of galvanized wire 3 meshes per inch, except for the bottom which is 2 meshes per inch, are provided for each animal. The

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food cup and water bottle are attached to opposite sides of the cage and are so made that there is very little spilling of food or water and practically no contamination of either with urine and feces. The cage stands on 3 inch legs.

The experimental animals are always taken from the normal stock colony on the 21st day of life, unless otherwise indicated. A minimum weaning weight of 35 gm. is allowed. The stock colony is maintained on McCollum's Diet I (whole ground wheat 67.5, casein 15, whole milk powder 10, butter fat 5.2, calcium carbonate 1.5, sodium chloride 0.8).

The experimental diets used in this work are unique in their simplicity and purity. They are slight modifications of Diet 519 previously introduced by one of us (1). First, the sucrose is no longer purified by recrystallization from 80 per cent alcohol since we have been unable to detect any effect upon the rat, due to this additional purification. Second, the casein is not made from fresh milk according to the directions of Van Slyke and Baker (2) because this involves the handling of such a large bulk of material. The procedure for the production of casein has been so modified as to meet the two demands: (1) high purity and (2) quantity production.

The following is the process adopted. 110 gm. lots of high grade curd casein are put in 3 liter Pyrex Erlenmeyer flasks and washed twice (for 2 days) with distilled water. This removes much soluble material. After draining the second wash water for several minutes 1300 cc. of 0.085 N NaOH are added along with 1 cc. of toluene and the flasks shaken during the next 24 hours. This gives a solution with about 7 per cent casein and relatively little other material. Six of these batches are strained into a tall 12 liter battery jar. Before the casein solution is added the stirrer and two acid-carrying tubes are set up so that the bent tubes feed a slow fine stream of acid into the most violently stirred liquid a few mm. from the stirrer. A 1 : 1 mixture of normal hydrochloric acid and normal acetic acid is used. The stirrer runs at 2000 R.P.M. Since about 700 cc. of the acid must be added for complete precipitation, the time of precipitation has been decreased by increasing the rate of addition of acid somewhat over that advised by Van Slyke and Baker. Acid flows from each burette at the rate of about 3 cc. per minute until the solution becomes white and begins

to increase in viscosity. The acid flow is then cut in half and continued until the dilution test shows almost complete precipitation. It is then reduced to a very slight dribble and the stirring continued for another half hour, when the dilution test will show complete precipitation. The total time of precipitation is almost 2 hours.

The mixture is now transferred to a 5 gallon bottle and washed with distilled water daily until the chloride test is negative or extremely faint. This washing requires about a week if there is thorough shaking with each washing. Alcohol is then added to the settled casein to bring it to about 50 per cent alcohol. This is settled and after the supernatant liquid has been siphoned off, the casein is transferred to three large closed filter funnels and drained. It is then transferred to tall settling jars and washed with alcohol twice or until the supernatant liquid is 90 to 92 per cent alcohol by volume.

Two such batches of casein are prepared at a time and these are transferred to a strong cloth bag which fits in a large glass Soxhlet extractor (7" diameter \times 21" tall); the last of the alcohol is drained off for 24 hours, and ether extraction begun. The ether washes out the alcohol, returns it to the 12 liter boiling flask where a fractionating column separates the ether from the dilute alcohol, and after 1 week of extraction the casein is ready to spread in pans to dry at 35°. This prolonged ether extraction is very important in producing a casein free from fat.

The casein thus prepared has not been injured by exposure to alkali (3) or high temperature (maximum temperature is the boiling point of ether). No known vitamins are present in detectable quantity. By producing the double batch weekly (1320 gm. of crude casein yielding about 1000 gm. of the pure air-dried product) a colony of 100 healthy rats can be maintained.

The salts are commercial products of C.P. or U.S.P. quality. McCollum's Salt Mixture 185¹ has been used. A trace of potassium iodide is added to the water.

The vitamin B (complex) is furnished by Northwestern pure dehydrated yeast which has been extracted for 48 hours with ether in a Soxhlet apparatus. About 1.5 per cent fat is removed by

¹ McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

this extraction, and the product is designated as Yeast 5. This is fed separately in Syracuse dishes.

Vitamins A and D are furnished by the non-saponifiable matter from high grade cod liver oil.² The non-saponifiable matter from 0.5 gm. of the oil is fed to each animal in a week. Experiments have proved that larger amounts are not beneficial. The rate of destruction of vitamin A in solution or in dry form when sealed in a partial vacuum is uncertain and probably varies greatly with small changes in condition. For this reason the storage of this material is not attempted but a new lot is made each week and fed during the following 7 days. For a group of forty animals the following procedure is employed: Alcoholic potassium hydroxide is prepared by dissolving 8 gm. of high grade KOH in 5.4 cc. of water and this solution is mixed with 40 cc. of purified 96 per cent alcohol. This product gives no color on boiling for 1 hour. The potassium hydroxide is decanted from the insoluble carbonate into a 200 cc. Erlenmeyer flask containing the 20 gm. of cod liver oil. After being refluxed for 1 hour on a steam bath, the whole is transferred to a 1000 cc. separatory funnel with 160 cc. of water and extracted once with 200 cc. of ether (U.S.P. for anesthesia).³ The extract is then washed free from alkali and concentrated to a few cc. so that about 2 drops carry the daily dose. The bottle is stored in the dark and the product retains its potency throughout the week. The dose is evaporated on the yeast, which is then mixed with distilled water and fed at once. The product is designated as Fraction AD.

A basal diet of the greatest simplicity was desired and everything was left out which was not definitely known to be necessary for the production of healthy, vigorous animals. Vitamin E was therefore omitted from the diet (which, accordingly, always produces sterile animals, readily curable). Some animals are now being fed vitamin E to determine whether this vitamin plays any rôle in the deficiency described here.

Distilled water has been given throughout all experiments.

In the planning of the diets some of the principles advocated by

² Patch cod liver oil, with a guaranteed vitamin A value, has been used throughout these experiments.

³ Merck's ether put up in the $\frac{1}{2}$ pound can has been used, so that it is perfectly fresh each week. No purification is necessary.

feeders of farm animals, but largely ignored by nutrition workers using the smaller animals like the rat, have been applied. Henry and Morrison (4) advise the following schedule for pigs: from weaning time until 100 pounds in weight, a nutritive ratio of about 1 : 4, from 100 to 200 pounds in weight a nutritive ratio of 1 : 5 to 6, and from this time on a nutritive ratio of 1 : 7. A similar schedule is followed in this laboratory for many experiments. At weaning date the animals are given a diet of nutritive ratio 1 : 3; when about 100 gm. in weight they are changed to a ratio of 1 : 5; when 175 gm. in weight (or when the growth curve is definitely flattened) they are changed to a ratio of 1 : 7. A diet with a ratio of 1 : 7 is considered good for maintenance of mature rats.

TABLE I.
Composition of Diets.

Diet No.	Pure casein	Sucrose	Salt Mixture 185.	Lard.	Nutritive ratio.	Salts per 100 calories.	Fat calories.	Calories per gm. of diet.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>gm.</i>	<i>per cent of total</i>	
550	24 0	72 1	3 9	0	1:3	1 0	0	3 85
550 A	16.0	80 1	3 9	0	1:5	1 0	0	3 85
550 B	12 0	84 1	3 9	0	1:7	1 0	0	3 85
560	30 1	45 1	4 8	20	1:3	1 0	37.4	4 81
560 A	20 0	55 1	4 8	20	1:5	1 0	37.4	4 81
560 B	15 0	60 1	4 8	20	1:7	1 0	37.4	4 81

The advantages of this gradual change in nutritive ratio are three: (1) Adequate protein for early growth is assured without taxing the kidneys in later life. (2) Even carefully purified casein is the chief source of organic impurities in the basal ration and this factor is greatly reduced by partial replacement of casein by sucrose as the animal grows older. (3) The cost of the purified casein is so great that it should not be used as energy food except for special experiments.

The quantity of salt mixture in the diet is also given a constant value, based on calories. In the absence of reliable data on optimum values, the arbitrary standard of 1 gm. of salt mixture per 100 calories of diet has been adopted.

In Table I the data are compiled for the diets used in this

investigation. The approximate physiological fuel values of 4, 4, and 9 are used for casein, sucrose, and lard⁴ respectively.

Description of the New Disease.

When the rat (either the Wistar or Long-Evans strain) is reared on Diet 550 (Diets 550 A and 550 B) supplemented by 0.65 gm. of ether-extracted yeast and the non-saponifiable matter (our Fraction AD) from 70 mg of cod liver oil daily, an abnormal, scaly condition of the skin is observed between the 70th and the 90th day of life. Later the tip of the tail may become inflamed and swollen, and the whole tail soon is heavily scaled and ridged. Hemor-

TABLE II

Summarizing Observations Made on Group of Animals Kept on Diet 550 + Fraction AD + Yeast 5 without Any Fat Supplement.

Rat No	Maximum weight	Weight at death	Age at first marked abnormality of feet or tail	Age at onset of decline in weight	Age at death
	gm	gm	days	days	days
2,897 ♀	150	108	77	147	188
28,110 ♀	160	115	84	133	239
28,104 ♀	138	105	70	126	253
28,108 ♀	160	110	77	168	253
2,899 ♂	182	108	77	140	190
28,103 ♂	197	115	70	140	225
28,111 ♂	163	Less than 137	84	112	145
28,109 ♂	178	115	70	140	244

rhagic spots may arise in the skin throughout the entire length of the tail. The swelling of the tip may gradually be replaced by a true necrosis, resulting in the loss of 1 to 3 cm. of the tail. The hind feet become red and somewhat swollen at times, in some cases with large scales over the dorsal surfaces. The hair on the back of the body becomes filled with dandruff. There is a tendency to lose the hair, especially about the face, back, and throat. Sores often appear on the skin.

⁴Swift's Silverleaf brand of lard has been used throughout these experiments.

The skin of the face especially seems to become sore at times and the irritation causes the animal to rub the face continually with his fore feet.

TABLE III.
Summarizing Some Observations Made on Animals of Table II.

Rat No.	Before death.	At autopsy.
2,897 ♀	Ovulating normally at onset of decline.	Feet, tail, and skin very scaly. No visceral fat. Kidney normal in appearance.
28,110 ♀	Ovulating normally at onset of decline. Blood in urine.	Feet, tail, and skin very scaly. No fat in body. Kidneys mottled.
28,104 ♀	Ovulating poorly (long cycles). Much blood in urine.	Tail and feet very heavily scaled. Loss of hair at several spots. No body fat.
28,108 ♀	Ovulating poorly at onset of decline. Much blood in urine. Tail black and dead for 3 cm. from tip.	Kidney spotted. Tail, feet, and skin heavily scaled. No body fat.
2,899 ♂	Prolapsed penis. Very much blood in urine. Loss of hair on back.	Bloody urine in bladder. Kidneys abnormal. Tail, feet, and skin scaly. No body fat.
28,103 ♂	Prolapsed penis. Loss of hair on back. Blood in urine.	Tail, feet, and skin scaly. No body fat. Kidneys abnormal, white. Hard concretion in bladder.
28,111 ♂	Bloody urine.	Tail congested and hemorrhagic in places. Yellow concretion in bladder.
28,109 ♂	Prolapsed penis. Much blood in urine. Loss of hair on back.	Feet and back scaly. Tail heavily ridged with hemorrhagic spots. Kidneys abnormal. Concretion in urethra. Loss of hair about eyes and nose.

Often the progress of the caudal necrosis is not unlike that described by Smith and Bogin (5). These workers produced experimental gangrene by partial fasting (underfeeding), but observed no marked changes (aside from those of the cartilage) in microscopic sections. In their animals the advance of the process was checked

when the same diet (containing 22 per cent of lard) was fed *ad libitum*.

In the present experiments, the early outward signs of an unhealthy condition of the animal are soon followed by a cessation of growth when the animal is about 25 per cent underweight in comparison with the controls receiving fat. A plateau in the weight curve is maintained over a period of a few weeks to several months, when the animal begins to decline and invariably dies, unless given a curative dose of fat. Table II gives some observations on animals which were allowed to die. It is seen from these data that a decline in weight begins about the 5th month of life and the animal dies within 3 or 4 months.

Further observations and notes are summarized in Table III. Excepting the skin and tail lesions, the most marked and uniform pathology is observed in the urinary tract and the kidney. There seems little doubt that this is an important factor in the death of the animal. Some animals have died without the development of visible lesions in the gross external appearance of the kidney and without the appearance of blood or protein in the urine. But it seems likely that the renal disorder may prove to be an important and fairly constant result of the dietary fat deficiency (a good experimental method for producing renal disorders). Prolapse of the penis was frequently noted in the late stages of the disease, and vesical concretions occurred in a few cases. Histological studies of the various organs will be made by colleagues of the authors and it is hoped that biochemical studies will give a more exact knowledge of the nature of the metabolic derangement.

The lesions seem to be essentially different from those characteristic of low vitamin A or low vitamin B. There is never a xerophthalmia and neither low vitamin A nor low vitamin B has been shown to produce the caudal necrosis and scaly skin found in the present experiments. In the next section of the paper it will be shown further that increases in the dose of vitamin A and vitamin B (complex) do not effect cures.

The loss of hair and the skin lesions remind us of the pellagra symptoms in the rat described by Goldberger and Lillie (6). The following description of the development of the pellagra-like condition in the rat is given by these authors (p. 1028):

"After a variable period following the arrest of growth already mentioned, there has been observed in many of the animals so fed a tendency for the lids of one or both eyes to adhere together, with, in some instances, an accumulation of dried secretion on the margins of the lids. At about the time or shortly after the appearance of this ophthalmia there has developed in nearly, if not quite, every one of the animals on the indicated diets, some loss of fur. This fur loss has in some begun in irregularly distributed patches. More commonly it has been observed to begin either at the side or over the top of the head, the sides or front of the neck, or in the

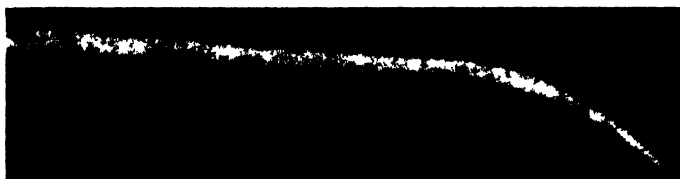


FIG. 1. Tail of Rat 28113 ♀. Diet 550 + 10 drops of lard daily. Entirely normal in appearance.



FIG. 2. Tail of Rat 28114 ♀. Diet 550 + 5 drops of glycerol daily. Glycerol gives no protection.

region of the shoulders. From these initial sites the depilation has extended and in some of the animals has led to almost complete denudation of the head, neck, and trunk. . . .

"With or without such loss of fur some of the animals have developed a dermatitis at one or more of the following sites: ears, front of neck and upper part of chest, forearms, backs of forepaws, shins, and the backs of the hind paws. This dermatitis, particularly as it has affected the paws, forearms, neck, and ears, has been sharply outlined and bilaterally symmetrical. To the eye it has differed somewhat with the site affected. The ears seemed definitely reddened and thickened with what appeared to be a

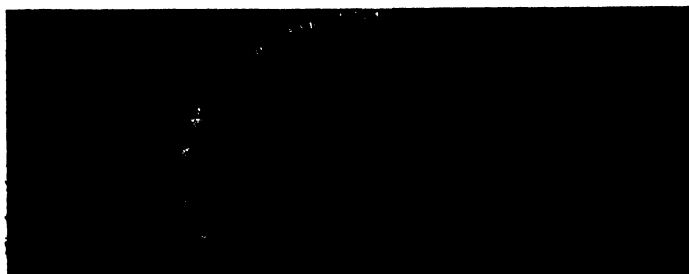


FIG. 3 Tail of Rat 2895 ♀. Diet 550 + the non-saponifiable matter from 10 drops of lard daily There is no protection from the disease.



FIG. 4. Throat and thorax of Rat 27121 ♀. Diet 550. Age 62 weeks. The animal was rapidly declining and died a week later.

yellowish incrustation of dried serum. In healing, desquamation took place, leaving the skin of the pinna with a polished, glistening, somewhat parchmentlike appearance. . . . In a few of the cases so far observed, the affected animals have presented a linear fissuring or ulceration at the angles of the

mouth. In a somewhat larger number there has been a lesion at the tip of the tongue, which first appeared as a small roughly circular grayish opacity or bleb, or as an ulceration which, in some went on to the formation of a localized yellowish slough "

Unlike pellagra the new disease affects the posterior portions of the body first. Necrosis of the tail has not been mentioned by Goldberger and his coworkers, while it is the most marked symptom of the new disease. Sores and ulcers seem common in the pellagra rat while they occur rarely in animals afflicted with the disease caused by low fat diets.

The most convincing evidence against the identity of the two diseases is the method of producing them. The pellagra diet contains 2 per cent of cod liver oil and 3 per cent of Crisco and must be deficient in the P-P factor, a normal constituent of whole yeast. With the addition of 9 per cent autoclaved yeast the pellagra-like condition disappears. But for the production of the new disease, all fat must be excluded from the diet, while 0.7 gm. daily of whole yeast powder does not protect the animal.

Figs. 2 to 4 show some of the lesions characteristic of the disease. Fig. 1 is a photograph of a tail kept normal by the addition of 10 drops of lard daily to the fat-free diet. Fig. 2 shows the swollen tip with a constriction about 2 cm. from the tip. The tail was finally severed at this point, while the 2 cm. end blackened before dropping off. Another conspicuous tail change is pictured in Fig. 3. Very heavy scales and ridges occur along almost the entire length of the tail, with hemorrhagic spots scattered over the surface. The loss of hair on the ventral aspect of the throat and thorax is shown in Fig. 4. The very dark spots are sores. This condition is not so common as the tail lesions and has been observed in those animals only which are in very bad condition and have but a few weeks to live.

In two of the older and more severe cases all four feet have become swollen and in one case the toes of the hind feet have developed sores and ring lesions apparently similar to those of the tail.

Comparative Growth and Ovulation with and without Fat.

When the young rat is reared on Diet 560 + 0.65 gm. of Yeast 5 + Fraction AD, growth is not greatly retarded during the first 60 days, but the animal is always somewhat subnormal in size and

reaches a plateau earlier than animals receiving fat. In most of the graphs presented here curves for averages of groups of animals have been given. This is permissible because the groups are quite uniform in weight. This method of presentation avoids the confusion of many intersecting curves.

There is an apparent sex difference in the results. Chart 1 shows that the curves for the males plateau when they have at-

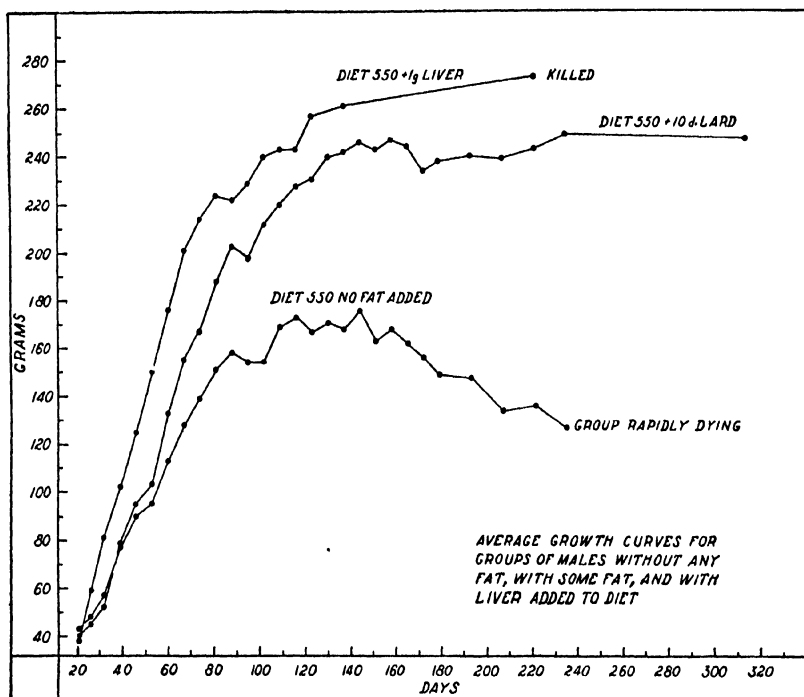


CHART 1.

tained only about 70 per cent of the weight of their controls receiving lard. Chart 2 shows that the curves for the females plateau when their weight is about 80 per cent of the maximum reached by animals receiving lard. Furthermore, when the males begin to decline their loss in weight is much more rapid than that of the females. But in the case of second generation animals (Chart 3) the females are greatly retarded, never quite weighing 70 per cent

as much as their controls at maximum weight. Liver seems always to improve the diet more than the addition of fats. An effort is being made to explain the exceptional value of liver as a supplement to these diets.

These data are in agreement with the findings of Evans and Burr (1) that diets containing 5 to 20 per cent of lard gave materially better growth than did diets containing no fat except 3 drops of cod liver oil.

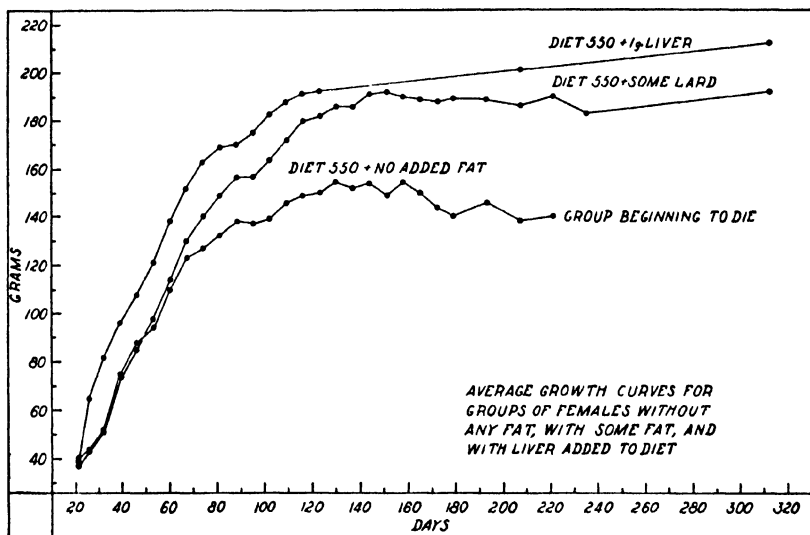


CHART 2.

The next step was to find out about what quantity of lard the rat must consume daily to be greatly benefited in growth and health. Chart 4 gives the average growth curves for females on Diet 560 (20 per cent lard) and Diet 550 + 10 drops of lard. 10 drops of melted lard weigh about 200 mg. and are therefore about 2 per cent of the total food consumed. It is evident that 2 per cent of lard is just as beneficial as 20 per cent. The animals receiving the smaller amount are entirely free from skin lesions or tail trouble.

Other groups of animals have been reared with 3 drops of cod liver oil as a source of vitamins A and D instead of Fraction

AD. This amount of fat in the diet is sufficient to prevent the decline and early death of animals. They will have some scales on the tail and feet but otherwise will be healthy for a period of a year. Two groups of animals receiving cod liver oil were changed to Fraction AD. One group had received no other fat besides the cod liver oil, while the other group consumed some lard in the diet. When the cod liver oil was stopped, the

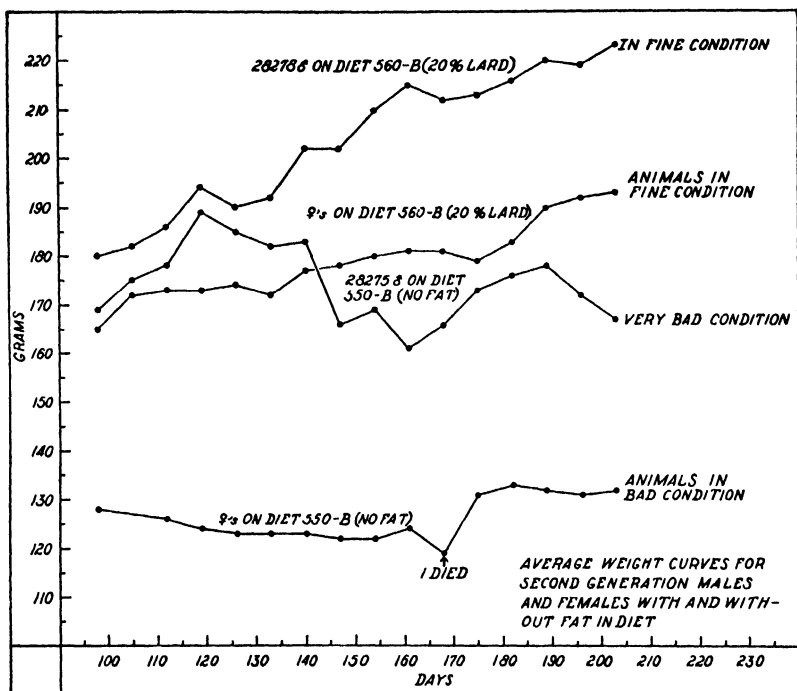


CHART 3.

former animals were on a fat-free diet. The gradual decline of the animals deprived of fat is shown in Chart 5. The male lost weight at a remarkable rate. The animals receiving lard were not adversely affected by the change from cod liver oil to Fraction AD.

Next, it was necessary to determine what part of the lard was responsible for the good nutritive effects observed. A standard procedure for saponification was followed. 100 gm. of fresh leaf lard were saponified for 1 hour on a steam bath in alcoholic potash

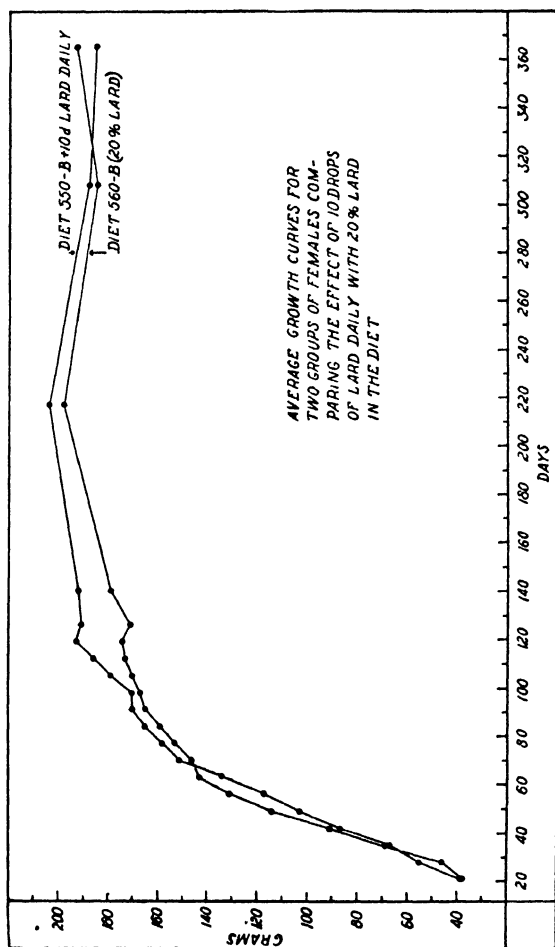


CHART 4.

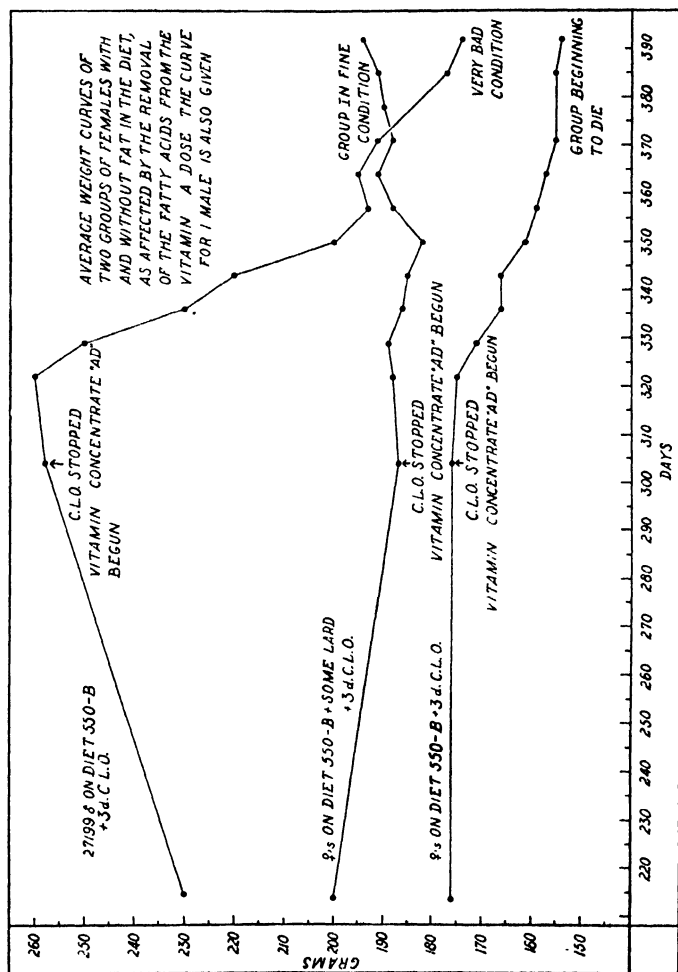


CHART 5.

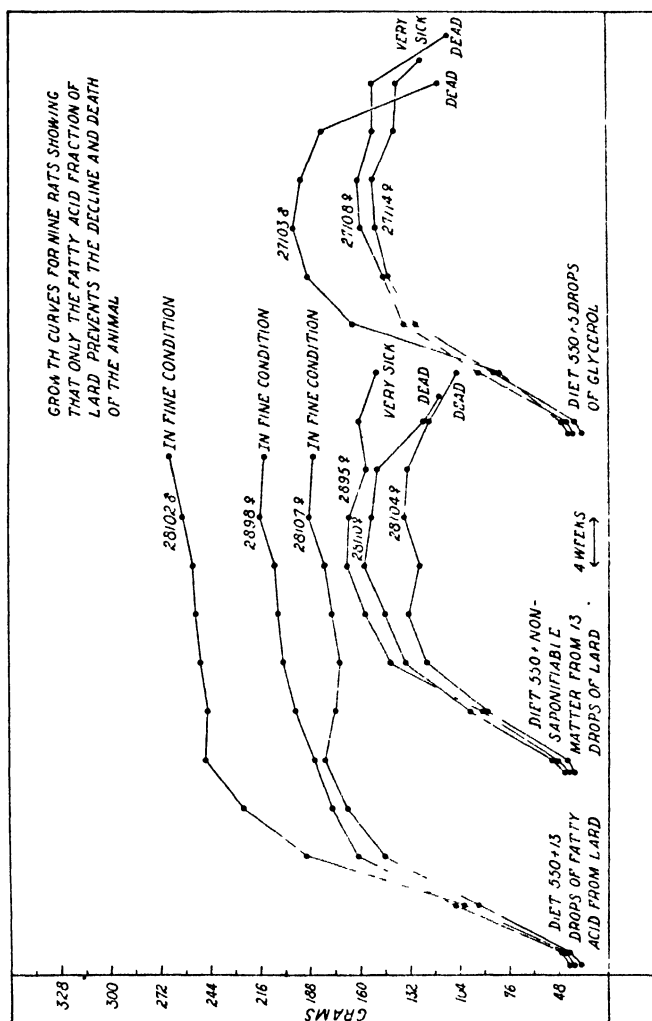


CHART 6.

(200 cc. of purified alcohol, 40 gm. of pure potassium hydroxide dissolved in 27 cc. of water). The hydrolysate was diluted with 1000 cc. of water and extracted three times with 700 cc. of U.S.P. ether. The fatty acids were liberated with an excess of hydrochloric acid, separated, and washed thoroughly. They were then freed from solvents and water by prolonged treatment *in vacuo* at 60° and were stored at 0°.

The non-saponifiable matter was washed free from alkali, concentrated, and then dried on the water bath at 60° *in vacuo*. The material was dissolved in ether for daily feeding.

Pure glycerol was used as the third fraction of lard and was fed in daily doses of 5 drops.

13 drops of melted fatty acids were fed daily to one group and the non-saponifiable matter from 13 drops of lard was fed to another group. The results are summarized in Chart 6. The fatty acids gave complete protection, while the glycerol and non-saponifiable matter did not affect the animals in any measurable way. They soon developed the characteristic skin and tail trouble and later declined and died unless a curative dose of fat was fed.

It should be noted that ovulation was recorded for many of the animals, and that the results lack uniformity. As a group, the fat-fed animals ovulate more regularly (4 day cycles) than those animals receiving no fat. But in many cases the animals will have ceased growing and will have developed serious tail and skin trouble before ovulation is affected. The ovulation histories are therefore omitted here because the appearance of the skin and the growth curves are more reliable measures of the condition of the animals.

Curing the Disease.

In one of the earlier experiments six females, which had developed tail lesions, and had ceased growing, were changed from Diet 550 B to Diet 560 B. Renewed growth was observed at once and the tail and feet of every animal became smooth and glossy. The emaciated animals soon were well covered with fat. It was thought that much of the gain in weight was due simply to storage of ingested fat.

A new experiment was then tried with the much smaller dose of lard, 10 drops daily. Four animals were chosen which were in

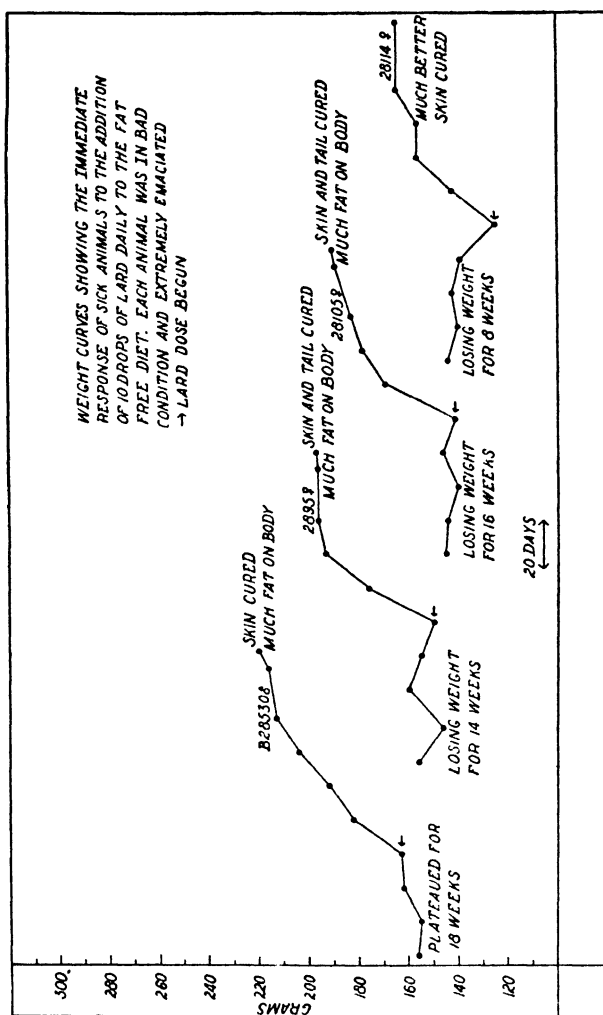


CHART 7.

very bad condition and could not have lived much longer. Their mates were already dead and these were the survivors from a total of 16 animals. The remarkable effect of this small addition of fat is shown in Chart 7. An immediate change in metabolism is indicated. These emaciated animals, which are from 40 to 60 gm. underweight, begin to grow and for a period of several weeks show a gain of about 2 gm. per day. The skin and tail become smooth and after 10 weeks complete cures are realized. The animals become normally fat and are in general good health. The cure is as spectacular as those cures produced by the well known vitamins. Similar cures have recently been made with even smaller doses of fat. These will be reported in a forthcoming paper.

Although it seemed improbable that there was an insufficiency of vitamins A and D in the dose of Fraction AD fed, two tests were made to prove the adequacy of the fraction. Three young animals were taken from stock mothers and put on Diet 550 + 0.65 gm. of ether-extracted yeast + 10 drops of lard daily. No Fraction AD was fed. These animals reached maximum weights of 73, 78, and 90 gm. After being on the diet 7 weeks all had xerophthalmia and on the 8th week one died, while the other two were killed because their eyes were closed and they were not eating. Their controls, which were receiving a daily dose of Fraction AD were growing normally and have shown no signs of deficiency at 10 months of age.

Then an attempt was made to cure sick animals by doubling the dose of Fraction AD. Four animals were used. All continued to decline in weight and become worse. Two died and the other two were saved by being returned to the normal dose of Fraction AD and fed a few drops of fat daily.

Similarly, two animals which were declining in weight and were badly diseased, were given a 30 per cent increase of yeast. They were then receiving about 0.88 gm. of dried yeast daily instead of 0.65 gm., the usual dose. There was no gain in weight and the feet and tails continued to grow worse. These animals would have died if a curative dose of fat had not been given.

DISCUSSION.

In 1920 Osborne and Mendel (7) took up the question of the requirement of animals for fat in the diet and concluded that "if

true fats are essential for nutrition during growth the minimum necessary must be exceedingly small." But their diets were only relatively low in fat since anhydrous ether extracted 0.24 per cent of solids and it is certain that meat residue, alfalfa leaves, and starch contain much fatty material which is non-extractable. In fact, it is impossible to conduct a fat-free experiment if corn-starch is used as a source of energy since it carries about 0.6 per cent of fatty substance which is within the granule and is non-extractable (8).

Since Osborne and Mendel (7) review the literature and the arguments on both sides of the question, this will not be repeated here. It is sufficient to say that these earlier experiments were not critical since either they were of very short duration or the diets contained appreciable amounts of fat.

About 3 years ago one of us began working with highly purified basal rations consisting of carefully prepared casein, sucrose, and salts. The basal ration might be said to be almost fat-free but the experiments were again made less critical for this specific problem by the addition of 3 drops daily of cod liver oil to supply vitamins A and D and the 700 mg. of yeast was not ether-extracted. Together, these two supplements added to the diet of each rat at least 70 mg. daily of ether extract, besides some non-extractable fatty substances in the yeast cell. Even with the ingestion of this much fat the animals were markedly inferior to their controls receiving 5 to 20 per cent of some well known fat (1). Fat was thus shown to be beneficial to the animal but not essential to long life and comparatively good health.

In a comprehensive paper, Krogh and Lindhard (9) demonstrate that there is a certain fat minimum as well as a carbohydrate minimum for the best utilization of foods. They postulate that when the respiratory quotient is above 0.9 there is a transformation of carbohydrates to fat and this gives rise to an extra expenditure of energy during rest. This effect would reach a maximum with a respiratory quotient of 1.0.

By the present work the authors have shown that if the young animal is subjected to a complete fat starvation over a period of several months, it develops a disease and soon dies. Whether this effect is caused by the strain of long continued fat synthesis suggested by Krogh and Lindhard or whether a special type of fatty

acid is required by the animal which it is unable to synthesize from the diet consumed, are unanswered questions. There is the further possibility that the ingestion of 200 mg. of fatty acids favorably affects the alimentary canal and improves the general well being of the animal. These problems are being studied now.

The fatty acid fraction of lard is fairly well known. Some workers have almost quantitatively accounted for all acids present with about the following distribution: stearic acid, 15 per cent; palmitic acid 25 per cent; oleic acid, 50 per cent; and linoleic acid, 10 per cent. Traces of arachidonic, linolenic, lauric, and myristic acids have been reported. Ellis and Isbell (10) show that the diet has a marked effect upon the distribution of the fatty acids in lard. If these well known fatty acids are responsible for the cures described (Chart 7), then we must assign to them a function far more subtle than the production of nine calories of energy per gm. burned. By their presence they have changed the entire economy of the animal, causing an increase in body weight equal to 10 times the weight of the acids consumed. The increase in weight is always accompanied by a return to normal health.

If the effect is not due to the ordinary fatty acids, then we must look for a new substance of the nature of an ether-soluble organic acid which must be present in exceedingly small amounts. This acid would be classed as a vitamin until its isolation permitted the assigning of a definite chemical formula and name. No conclusion can be drawn from the data at hand and for the present we will speak of this dietary deficiency as due to the absence from the diet of the acids present in fats.

SUMMARY.

1. New diets of high purity and extremely low in fat have been devised.
2. A new deficiency disease involving caudal necrosis in the rat has been produced by the careful exclusion of fats from the diet.
3. This disease is readily prevented or cured by the addition of 2 per cent of fatty acids to the diet.
4. The non-saponifiable fraction of fats and glycerol are ineffective for the curing of the disease.
5. As little as 3 drops of fat fed daily has a measurable effect upon the animal.

6. With fat-free basal rations, storage fat can be almost entirely eliminated and it seems probable that the amount of body fat can be controlled over a wide range by the addition of minute quantities of fat to the diet.

The authors wish to express their thanks to Professor J. Arthur Harris and Professor C. M. Jackson for their interest and for their assistance in the preparation of the paper.

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DETERMINATION OF SUGAR IN BLOOD.

I. OBSERVATIONS UPON BENEDICT'S ALKALINE COPPER SOLUTION.

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The work of Benedict (1, 2) and Folin (3, 4) reveals an effect of sulfite in alkaline copper reagents that seems to have been but partially appreciated heretofore. Benedict attributed the somewhat lower values for blood sugar, which resulted with his earlier copper reagent (2), to the presence in the reagent of large amounts of sodium citrate. Incidentally, the reagent also contained sodium sulfite, and it appears that, by the incorporation of citrate alone, higher values for blood sugar are obtained. Folin (3) noticed that Benedict's citrate-sulfite reagent, after standing for some time, gave values which approached the higher Folin-Wu values. He attributed this phenomenon to the oxidation of the sulfite, indicating that the low values of Benedict's reagent are referable to the sulfite rather than the citrate.

During an extensive investigation of alkaline copper solutions, I have had occasion to make use of Benedict's sulfite effect in a variety of experimental copper mixtures. The result, without exception, has been a lowering of the apparent blood sugar values, regardless of the nature of the other components of the alkaline copper solutions. Thus, blood sugar values 10 to 20 per cent lower than similar Folin-Wu values were always obtained with sulfite-copper reagents which contained malate, glycine, salicylate, or pyridine in place of tartrate, or with reagents in which tartrate was combined with these substances or with phthalate, phosphate, etc. Moreover, the Folin-Wu copper reagent gave similar low values when proper amounts of sulfite were added to it. In these experiments, I used fresh solutions of anhydrous

sodium sulfite or bisulfite. The amounts selected for addition to the copper mixtures were those which gave appreciable increases in color production when the reagent was heated with glucose, but which gave only very small blanks.

The effect of sulfite is a general one. With no copper mixture is it entirely inactive, and it always causes intense reduction by itself, if enough be added to a copper mixture. On the other hand, the sensitivity of alkaline copper mixtures to reduction by sulfite is quite variable. For example, the Folin reagent (3) is so sensitive that very small amounts of sulfite give a marked blank. Decreasing alkali concentration appears to increase the sensitivity of copper reagents to reduction by sulfite. Reagents contain-

TABLE I.

Type of copper mixture.	Increase in color.	Na_2SO_3 in copper solution.
	<i>per cent</i>	<i>per cent</i>
Phosphate-tartrate.....	45	0.5
Phthalate-tartrate.....	45	0.5
Malate.....	65	1.0
Salicylate.....	55	0.5
Pyridine-tartrate.....	45	0.5
Glycine-tartrate*.....	25	0.2
Alanine-tartrate*.....	20	0.2

The Folin-Wu technique and Folin acid molybdate solution were used.

* Indicates copper solutions equivalent to Benedict's latest copper reagent (5). With these Benedict's technique was used.

ing malate or glycine are less sensitive to sulfite reduction than those containing tartrate.

When we add to a copper reagent smaller amounts of sulfite, which in themselves cause little or no blank, there usually results a marked increase in the amount of reduction by 0.2 mg. of glucose. In Table I, I have given these increases for a few copper mixtures, but with no pretense of great accuracy. Even greater increases occur with other amino acid mixtures, less alkaline mixtures, and certain ammoniacal mixtures, when the original reduction is very small. Sulfite increases reduction disproportionately for various concentrations of glucose. In mixtures such as Benedict's latest copper solution, it increases the reduction by

0.2 mg. of glucose more than that by 0.4 mg. of glucose, thereby bringing the depth of color into direct ratio with the amount of glucose (over this range).

TABLE II.

The results are expressed in mg. per cent.

Type of reagent.	Time after diluting.	Blood sugar.	
		Experimental reagent.	Folin-Wu.
	<i>min.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Glycine-sulfite.	1	94	108
	5	92	
	10	90	
	15	88	
	30	81	
	35	80	
Without sulfite.	5	105	
	10	107	
	35	107	
Glycine-tartrate-sulfite.	5	91	108
	10	90	
	45	64	
Without sulfite.	5	99	
	10	100	
	40	99	
Malate-sulfite.	5	95	108
	10	93	
	35	87	
Glycine-sodium nitrate-sulfite (in concentrations equivalent to Benedict's reagent).	5	70	88
	10	68	
	15	67	
	40	60	

Folin acid reagent and technique were used. Bubbles were carefully avoided in the colorimeter cups.

With these facts and some unpublished data in mind, it seemed that an ideal copper mixture, for securing low blood sugar values, should result, if some amino acid, sodium sulfite, and a large concentration of an inactive salt were part of the alkaline copper

solution. Such mixtures were prepared from glycine,¹ and were being studied at the time that Benedict's new alanine-tartrate-sulfite reagent was announced (5). This reagent duplicated my results so completely that the development of the glycine reagent was unnecessary.

However, there was another reason for my hesitation to recommend the use of a similar sulfite-containing reagent. It occurred to me that the apparently low values for blood sugar, given by this class of reagents, might be due to an unequal and deceptive

TABLE III.

The results are expressed in mg. per cent.

Time after dilut- ing <i>min.</i>	Sample No											
	1	2	3	4	5	6	7	8	9	10	11	12
5	76 5	90 5	75 5	53 5	74 5	72 5	74	79	71		125	72 5
10	72								70	66	119 5	
15									68 5			
25		84 5										66.5
30						62						
35	68		68	50	68		65 5	72				
40	61	80							63.5		104	
50										57 5		
60				48	67 5							

Sample 8 was plasma of Sample 9.

Sample 9 without sulfite in reagent; 73 mg. per cent at 10 minutes, 73 mg. per cent at 40 minutes.

Sample 10 without sulfite in reagent; 73 mg. per cent at 5 minutes, 73 mg. per cent at 50 minutes.

fading of the colors in the standard and unknown, rather than to an increased specificity for glucose. I investigated the stability of the colors with this hypothesis in mind and found that all of the sulfite-containing reagents did show the postulated fading. (See Table II for examples.)

Considering the similarity between some of these experimental reagents and the new Benedict reagent, it would be rather sur-

¹ I have subsequently come to the conclusion that other amino acids, notably glutamic acid and α -aminoisobutyric acid, may be more desirable components of copper mixtures.

prising if the latter did not show the same defect. I examined the stability of the colors in his method with results similar to those in Table III. Fading is present though slow enough to escape casual detection. The solutions were allowed to stand in the Folin-Wu blood sugar tubes until the time for reading. No question of unequal accumulation of gas bubbles beneath the plungers can be thought of, since this condition was carefully avoided. By using all glass cups, one can detect the very smallest bubbles which often collect upon the floor of the cup, as well as those under the plunger.

In order to be certain of these experiments, I have repeated them with new reagents made from samples of pure alanine, from various sources, and from several samples of bisulfite. In addition, I used, at different times, both the acid molybdate solution described by Benedict and the new acid molybdate solution of Folin (3). The results were always similar. Hence, the inequality of fading in standard and unknown is hardly due to impurities in my reagents, nor to any detectable error of technique. While I cannot say to what extent these facts invalidate Benedict's method, I can scarcely feel as confident of it as of the Folin-Wu and Folin methods, where such unequal fading does not occur. It may be argued that errors would be small if readings were made at once, but there remains the uncertainty of how much fading occurs before it is possible to compare the solutions in a colorimeter, and with this the question of the true blood sugar values. At least one may conclude that the observed values are too low. In fact, I have frequently encountered negative values for hydrolyzable sugar of blood by Benedict's new method, when, at the same time, other methods proved the presence of appreciable amounts of such sugar (6). There is another disturbing factor in that the rate of fading appears to be different for different samples of blood filtrate. Incidentally, all of my filtrates were made with lithium oxalate cloth as anticoagulant and were ascertained to be neutral.²

Other reducing substances can exert an effect like sulfite. The replacement of it by very dilute, freshly prepared solutions of

² I wish to acknowledge the assistance of Fay Sheppard, of the University of Oklahoma Medical School, and of J. F. Gordon, of the University Hospital, in the preparation of some of my filtrates.

semicarbazide hydrochloride or phenylhydrazine hydrochloride increases the reduction by glucose, giving about the same values for blood sugar as sulfite reagents. I have also experimented somewhat with tellurite, arsenite, phosphite, formate, hydrazine sulfate, hydroxylamine sulfate, selenite, and nitrite for similar purposes. The last three substances cause remarkably rapid fading of the blue colors. The results I have obtained with these

TABLE IV.

The results are expressed in mg. per cent.

Sample No.	Time after diluting.	0.2 mg. standard.	Blood filtrate.		0.4 mg. standard.
			A	B	
	<i>min.</i>				
1	5	99	99.5	101	194
	30	94.5	93.5	102	227
2	5	100.5	74	70.5	207
	45	95.5	64	70.5	222
3	5	106.5	102.5	107	200
	35	104	91.5	108	213
4	5	108.5			
	40	100			
5	5	105			
	40	99.5			
Acid molybdate solution.		2 cc.	2 cc.	4 cc.	2 cc.

The 0.2 mg. standards and Blood Filtrate B were read against a 0.2 mg. standard with which 4 cc. of acid molybdate solution were used. Other readings were made against a 0.2 mg. standard with 2 cc. of acid molybdate solution.

substances are too confusing to recommend their substitution for the sulfite of Benedict's reagent.

The color of reduced acid molybdate solutions is not permanent, no matter what copper reagent is used. For example, the color in the Folin method fades as much as 20 per cent in the course of an hour or more. I have been interested in making these colors more permanent and have found, in the case of tartrate reagents,

that heating the acid mixture for several minutes, before diluting, accomplishes this result. When I attempted to use this procedure with Benedict's copper reagent and Folin's acid reagent, complete decolorization resulted. In some experiments upon the original copper reagent of Ost, I noticed that fading was marked unless a definite excess of acid molybdate solution was used. By

TABLE V.

The results are expressed in mg. per cent.

Sample No.	Time after diluting.	Original filtrate.		Fermented filtrate.		Fermented filtrate + 86 mg. per cent glucose.	
		Folin.	Benedict.	Folin.	Benedict.	Folin.	Benedict.
1	min.						
	5			11.5	10.5	96	93
	45			11	8.5		76
2	10	68	72.5	10.5	14	92.5	73
	45		63	10	11		63
3	5	54	60	11.5	13	96	95
	45		53	12	12		82
4	10	60.5	59.5	8.5	9.5	93	86
	45		51.5	8.5	9		76.5
Fermented filtrate + 82 mg. per cent glucose.							
5	5	88	78.5 (90.5)	10	14 (13.5)	91	86.5 (95.5)
	45	90	72 (92.5)	10.5	11.5 (11)	91.5	82 (98)
6	10	84	80.5 (90)	9.5	15 (14)	94	86.5 (96)
	45	85	76 (89.5)	9.5	12.5 (13.5)	93	81.5 (97)

The figures in parentheses represent duplicate analyses with 4 cc. of acid molybdate solution.

increasing the acid molybdate solution from 2 to 4 cc. in Benedict's method, the unequal fading of unknown and standard seems to be eliminated (Table IV).

The larger amount of acid molybdate solution diminishes the fading in 0.2 mg. standards. In the case of 0.05 mg. standards, which are finally diluted to 10 cc., 2 cc. of the acid solution cause less fading. I have also noted that fading is comparatively more rapid in the more dilute glucose standards. Hence, proportion-

ality changes somewhat as the blue colors fade. (See last column of Table IV.)

It is possible to question the validity of Benedict's new criterion for blood sugar reagents. His results with fermented blood filtrate, before and after the addition of glucose, may be due somewhat, if not entirely, to unequal fading. If, for example, the fading in each case is 10 per cent of the color over a given time, then 9 mg. per cent of determined residual reducing material would actually represent 10 mg. per cent, and after 100 mg. per cent of glucose have been added, the total would appear as 100 instead of 110 mg. per cent. I examined a few fermented filtrates to determine what actually occurs. The results are recorded in Table V. The fermented filtrates show an average change of 1.5 mg. per cent, and the fermented filtrates plus glucose, about 7.5 mg. per cent in 30 minutes. If similar changes occur in the interval between the addition of the acid molybdate solution and the comparison in the colorimeter, Benedict's results would be explained.

In the first four experiments I used the entire technique of Benedict, including his method of fermentation, except that the fermentation of the third and fourth samples was by the Folin-Svedberg method, with washed yeast. In the last two experiments, I used Folin acid reagent. The figures in parentheses represent duplicate analyses, with 4 cc. of acid molybdate solution. These figures show that the original blood sugar values are close to the Folin values, and also that added glucose is apparently recovered from fermented filtrates. I have made too few determinations with the larger amount of acid molybdate solution to recommend its use without further trial.

SUMMARY.

A study of the effect of sulfite upon alkaline copper reagents demonstrates that Benedict's new blood sugar method does not give true blood sugar values. The use of 4 cc. of Folin's acid molybdate solution is suggested as a possible modification.

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DOES CABBAGE FED TO RABBITS INCREASE SERUM CALCIUM?

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In her studies on the variations of serum calcium in rabbits, Culhane (1) raised the question of there being a specific calcium-raising substance in cabbage. She found that the feeding of cabbage temporarily raised the blood serum calcium. The first experiment included a group of six rabbits. Following the feeding of cabbage, blood samples were analyzed at intervals of 2 hours for a period of 6 hours. The average calcium estimations were as follows, the figures given being in mg.

	10.00 a.m.	12.00 noon.	2.00 p.m.	4.00 p.m.
Average of controls.....	13.39	14.49	13.54	13.04
Change from initial.....		+1.10	+0.15	-0.35

Two control experiments were carried through: the first against cabbage, water alone being given the day before, and the second against cabbage and water, yielded the following results.

	10.00 a.m.	12.00 noon.	2.00 p.m.	4.00 p.m.
Control 1 average.....	12.76	12.54	12.15	11.83
Change from initial.....		-0.22	-0.61	-0.93
Control 2 average.....	12.81	13.11	12.09	11.17
Change from initial.....		+0.30	-0.72	-1.64

In repeating these experiments on a large scale using young rabbits, she found the specific effect of cabbage was absent. No

tables were presented indicative of these results. A statement, however, follows to the effect that when serum calcium is high it is not easily changed, whereas when it is low it may be increased by a variety of means. This may account for the results of the third experiment. This consisted in bleeding twice then feeding cabbage and drawing blood twice subsequently for calcium determinations. These results are as follows:

	9.30 a.m.	11.30 a.m.	1.30 p.m.	3.30 p.m.
Average calcium.....	14.12	13.44	15.34	14.12
Change from initial.....		-0.98	+1.22	
		Fed cabbage.		

As a result of this last experiment, Culhane seems to have definitely decided that cabbage raises the serum calcium of rabbits. If this were true it would suggest that cabbage contains a substance analogous to that found in the parathyroid gland and cabbage should be added to the group of substances, hormones, found in the vegetable kingdom.

Because of this possible important association, the specific calcium action of cabbage was restudied. It was considered advisable to investigate the factor of hemorrhage since the experiments controlling this factor are different from those of the original ones described. In Culhane's experiments controlling the factor of hemorrhage there was an initial bleeding followed by another in 4 hours. The second determination shows a fall in serum calcium but the major experiment consisted of four bleedings in 6 hours. On the assumption that 5 cc. of blood are required for the analysis, the control animals each lost 10 cc. of blood, whereas the experimental animals lost 20 cc. or twice as much blood. The work of Clark (2), Moritz (3), and Stewart and Percival (4) as corroborative of the effect of hemorrhage on serum calcium, cannot be used for the following reasons: Clark used only one rabbit to control the effect of frequent hemorrhage on the blood and serum calcium. In Clark's study the fall in plasma calcium is not as consistent as one would be led to believe and since Culhane's determinations are on serum calcium, it is obvious that Clark's results cannot be used as conclusive evidence to establish the in-

fluence of hemorrhage on serum calcium in this work. Likewise in Moritz's experiments the animals were bled every 7 hours. Stewart and Percival on the other hand induced acute severe hemorrhage as indicated by a reduction in hemoglobin from 20 per cent to 66 per cent of the normal control.

The present study was divided into several phases in which the controls played the most important part. It was divided into two groups—the short and long term experiments.

TABLE I.
Influence of Cabbage upon Serum Calcium in Rabbits.

Time.	Hb. percentage of initial value.	Ca	Time.	Hb. percentage of initial value.	Ca
Rabbit 1; weight 1.5 kilos.			Rabbit 2; weight 2.7 kilos.		
Group 1 a. Short term experiment; control; 2 hourly bleedings.					
		<i>mg. per 100 cc.</i>			<i>mg. per 100 cc.</i>
10.00 a.m.	100	12.9	10.00 a.m.	100	11.6
12.00 n.	124	13.7	12.00 n.	105	11.9
2.00 p.m.	117	12.1	2.00 p.m.	96	11.2
4.00 p.m.	107	12.4	4.00 p.m.	109	11.8
Rabbit 3; weight 1.8 kilos.			Rabbit 4; weight 1.7 kilos.		
Group 1 b. Short term experiment; cabbage; 2 hourly bleedings.					
10.00 a.m.*	100	11.9	10.00 a.m.*	100	12.2
12.00 n.	72	12.8	12.00 n.	95	12.4
2.00 p.m.	71	12.0	2.00 p.m.	80	12.4
4.00 p.m.	72	14.4	4.00 p.m.	91	12.7

* Bled, then fed cabbage.

Short Term Experiments.—Group 1 a, effect of 2 hourly bleedings on hemoglobin and serum calcium for 6 hours. Group 1 b, effect of 2 hourly bleedings on hemoglobin and serum calcium for 6 hours with cabbage diet.

Long Term Experiments. Hemoglobin and Calcium Studies.—Group 1 a, effect of cabbage and daily bleeding. Group 2 a, effect of fasting for several days followed by cabbage. Group 2 b, effect of fasting for several days followed by oats and carrots.

TABLE II.
Influence of Cabbage upon Serum Calcium in Rabbits.

Day	Hb, percentage of initial value	Ca	Day.	Hb, percentage of initial value	Ca	Day.	Hb, percentage of initial value	Ca
Rabbit 1 weight 1 2 kilos			Rabbit 2, weight 1 5 kilos			Rabbit 3, weight 1 42 kilos		
Group 1 a Long term experiment; cabbage								
		mg per 100 cc			mg per 100 cc			mg per 100 cc
1	100	9 7	1	100	10 3	1	100	11 5
2	91	12 7	2	88	11 5	2	92	13 1
3	74	11 5	3	70	10 9	3	70	12 0
4	70	11 4	4	65	9 1	4	65	12 9
5	58	13 0	5	56	11 0	5	65	13 0
6	58	11 7	6	56	9 2	6	64	12 6
Rabbit 211 weight 4 2 kilos			Rabbit 212 weight 1 0 kilos			Rabbit 213, weight 4 0 kilos		
Group 2 a Long term experiment; fasting followed by cabbage								
1	100	20 9	1	100	12 4	1	100	14 7
2	92	15 1	2	117	10 9	2	92	14 1
3	87	14 5	3	99	12 1	3	75	16 3
4	70	13 6	4	77	14 0	4	76	12 9
5	71	14 1	5	70		5		13 9
6	60	15 7	6	70	14 5	6		
7	Fed cabbage		7	Fed cabbage.		7	Fed cabbage.	
	56	15 2		58	15 6		73	13 8
8	73	15 2	8	61	17 5	8	70	14 6
9	67	14 5	9	57		9	65	10 9
10	77	10 1	10	49	15 3	10	62	13 8
Rabbit 4, weight 2 0 kilos			Rabbit 5 weight 1 95 kilos			Rabbit 6, weight 2 5 k los		
Group 2 b Long term experiment; fasting followed by oats and carrots								
1	100	16 6	1	100	14 1	1	100	12 9
2	100	13 5	2	100	13 2	2	100	14 0
3	79	14 8	3	93	14 1	3	92	14 8
4	73	16 0	4	89	15 6	4	92	15 2
5	74	15 8	5	92	13 9	5	89	16 4
6	Rest period.		6	Rest period.		6	Rest period.	
7	70	15 0	7	89	14 4	7	76	15 5
8	Fed oats and carrots		8	Fed oats and carrots.		8	Fed oats and carrots.	
	69	14 5		92	14 0		80	16 0
9	65	12 8	9	77	13 4	9	68	14 7
10	45	12 2	10	60	14 5	10	50	12 5

TABLE II—Continued.

Day.	Hb, percentage of initial value	Ca	Day.	Hb, percentage of initial value	Ca	Day.	Hb, percentage of initial value	Ca
Rabbit 7, weight 2 0 kilos			Rabbit 8, weight 2 5 kilos.			Rabbit 9, weight 2 5 kilos.		

Group 3 a Long term experiment; acute hemorrhage and fasting.

		mg per 100 cc			mg per 100 cc.			mg per 100 cc.
1	100	12 0	1	100	13 8	1	100	13 8
	Bled 30 cc.			Bled 28 cc.			Bled 33 cc.	
2	73	12 3	2	64	12 1	2	72	12 3
3	69	11 6	3	62	12 2	3	73	12 0
4	66	11 6	4	64	11 6	4	70	12 0
5	61	12 0	5	58	11 8	5	69	11 8
6	65	13 8	6	62	12 2	6	65	12 8
7	Rest period.		7	Rest period.		7	Rest period.	
8	71	11 6	8	62	12 8	8	72	11 2
Rabbit 41, weight 2 0 kilos			Rabbit 51, weight 2 2 kilos					

Group 3 b Long term experiment; acute hemorrhage and cabbage.

1	100	11 3	1	100	11 7			
	Bled 27 cc.			Bled 30 cc.				
2	58	9 4	2	57	9 8			
3	74	11 7	3	87	11 9			
4	56	12 3	4	55	11 6			
5	49	11 4	5	44	13 5			
Rabbit 100, weight 2 6 kilos			Rabbit 101, weight 1 0 kilos			Rabbit 102, weight 2 0 kilos		

Group 4 a. Long term experiment; primary acute hemorrhage with cabbage diet.

1	100	13 3	1	100	11 9	1	100	14 2
	Bled 35 cc.			Bled 25 cc.			Bled 30 cc.	
2	75	13 1	2	56	9 8	2	84	14 3
3	40	12 1	3	61	13 0	3	61	14 7
4	40	11 9	4	61	13 9	4	57	14 7
5	50	13 4	5	56	14 2	5	57	14 9
6	49	15 1	6	62	14 0	6		
7	49	Lost.	7	64	15 3	7		14 2
8	75	16 3	8	77	15 1	8	61	15 0
9	52	15 7	9	66	13 9	9	61	14 7
10	56	16 0	10	57	14 8	10	60	13 3

TABLE II—*Concluded.*

Day.	Hb, percentage of initial value.	Ca	Day.	Hb, percentage of initial value.	Ca
Rabbit 10; weight 1.9 kilos.			Rabbit 11; weight 1.8 kilos.		
Group 4 b. Long term experiment; primary acute hemorrhage and cabbage followed by oats and carrots.					
		<i>mg. per 100 cc.</i>			<i>mg. per 100 cc.</i>
1	100	11.3	1	100	11.8
	Bled 30 cc.			Bled 30 cc.	
2	71	10.8	2	70	12.2
3	66	11.8	3	68	12.5
4	67	10.1	4	72	10.1
5	63	11.3	5	68	11.9
6	62	9.8	6	62	9.3
7	Rest period.		7	Rest period.	
8	53	9.2	8	53	10.7
	Fed oats and carrots.			Fed oats and carrots.	
9	65	10.0	9	75	10.5
10	57	9.4	10	64	10.4
11	55	9.3	11	75	11.4
12	58	9.3	12	70	11.3
13	57	9.4	13	75	11.4

Group 3 a, effect of acute hemorrhage and fasting. Group 3 b effect of acute hemorrhage and cabbage. Group 4 a, effect of acute hemorrhage and cabbage. Group 4 b, effect of acute hemorrhage and cabbage followed by oats and carrots.

Normal adult rabbits never previously used, weighing about 2 kilos, were placed in separate cages. Determinations were made upon blood samples drawn from the ear vein. Hemoglobin determinations were made by the Newcomer method. The serum calcium estimations were carried out by the Clark and Collip modification of the Kramer-Tisdall method.

The short term experiments (Table I) are actual duplications of those of Culhane. The control animals received only water. The second group received cabbage. It was found that the control animals during the short period of experimentation demonstrated rises in the serum calcium as frequently as falls. The animals receiving cabbage likewise showed varying results. The results of this group indicate that the statement that cabbage has a specific calcium-raising substance is not borne out.

The long term experiments (Table II) were carried out in order to see whether cabbage has a delayed effect.

Group 1 a.—These rabbits received cabbage throughout the entire experimental period of 5 days. The results demonstrate that daily variations in the serum calcium in rabbits are common. Rabbit 1 has a rise in calcium whereas Rabbits 2 and 3 show practically no change. The hemoglobin during this period shows the effect of daily bleedings. Hemoglobin content fell from 40 per cent to 50 per cent in 5 days.

Group 2 a.—These animals received only water for from 5 to 7 days. They were then fed cabbage for a period of 6 days. Rabbit 211 had an unusually high initial serum calcium of 21 mg. On the 1st day it fell to about 15 mg. and then remained practically constant. After being fed cabbage the serum calcium fell to about 10 mg. In Rabbit 212 after an initial fall on the 1st day of fasting the serum calcium rose to over 14 mg., and when fed cabbage the calcium continued to rise to 17.6 mg., later falling to 15.3 mg. Rabbit 213 had practically a constant serum calcium content and when fed cabbage had a sharp loss of 3.5 mg. followed by a sharp return. The control group during fasting showed the same irregular results. Rabbits 4 and 5 had a rather constant serum calcium whereas Rabbit 6 showed an increase. When these rabbits were fed oats and carrots the serum calcium fell. In both groups daily bleeding produces a marked reduction in hemoglobin and in spite of this there are prominent increases in the serum calcium.

Groups 3 a and 3 b.—In these groups acute hemorrhage was produced and its effect on the serum calcium during fasting (Group 3 a) and on a cabbage diet (Group 3 b) was studied. The animals were bled approximately 30 cc. Those receiving cabbage presented an initial fall of about 2 mg. following the acute hemorrhage with a subsequent rise to the normal level or higher. Those that received no cabbage showed practically a constant serum calcium. In these rabbits after the initial fall in hemoglobin it remained practically constant.

Groups 4 a and 4 b.—Rabbits receiving cabbage were severely bled (Group 4 a) and were controlled by similar ones fed later with oats and carrots (Group 4 b). In the first group Rabbits 100 and 101 had moderate falls followed by high serum calcium on the 3rd to 5th

day and then remained high. Rabbit 202 had no change at all. The second group showed an irregular fall during the cabbage feeding. In one case, Rabbit 11, with carrots substituted for cabbage there is a rise in serum calcium.

There is a prominent irregularity in different groups of rabbits used at the same time and for the same purpose. For example in Group 4 a, the rabbits receiving cabbage have an entirely different picture from those in Group 3 b.

DISCUSSION.

In this study a large number of rabbits were used. The conditions were constant except for the experimental factor involved. Although there are occasional suggestions that cabbage may have an effect in raising the serum calcium, they are too vague to state definitely that cabbage has the same effect as parathyroid hormone on serum calcium. In most cases during fasting with daily bleeding there is a fall in serum calcium. Daily bleedings in rabbits have a severe effect. The 5 cc. that are necessary for the determination of calcium may possibly interfere with a consistent effect produced by cabbage.

CONCLUSION.

As a result of this study it is impossible to state that cabbage has a definite calcium-raising substance.

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**A METHOD FOR THE PREPARATION OF GLYCOGEN
AND A STUDY OF THE GLYCOGEN OF THE
ABALONE, HALIOTIS RUFESCENS,
SWAINSON.***

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The methods by which glycogen is commonly prepared involve treatment with hot concentrated alkali. It may be questioned, therefore, whether the product thus obtained is glycogen as it occurs in the tissues or a derivative formed by the action of the strong alkali. The question assumes new importance now that it has been shown clearly that glycogen, at least from certain sources, contains phosphoric acid (1). It seems important, therefore, to determine whether the phosphoric acid content reported is the true content or whether phosphoric acid is lost in the process of preparation, for phosphate plays some sort of rôle in the intermediary metabolism of carbohydrate in muscle, although opinions differ concerning the nature of this rôle (2). In the present paper a method is described for preparing glycogen from the abalone, a local edible mollusc, without the use of powerful reagents, and analyses of the phosphoric acid and ash content of the glycogen thus prepared are recorded.

* Since the abalone meat was bought in a butcher shop, the species from which it was derived was not definitely determined. However, *Haliotis rufescens*, Swainson, the red abalone, is the one commonly used for food about San Francisco. Other species that are utilized in California either for food or for mother-of-pearl, etc., are *Haliotis fulgens*, Phil., the green abalone, *Haliotis wallelensis*, Stearns, the northern green abalone, *Haliotis cracherodii*, Leach, the black abalone, and *Haliotis corrugata*, Gray, the pink abalone (cf. Thompson, W. F., *Calif. Fish and Game*, 6, 45 (1920); and Goode, G. B., *The fisheries and fishing industries of the United States*, 2, sect. 5, Washington, 623 (1887).

The question of the phosphorus content of glycogen is also of interest in connection with the question of the ash content. Obviously, if phosphorus is regularly present, it should be impossible to obtain ashless preparations, yet many investigators have reported obtaining preparations free from ash, for example Gatin-Gruzevska (3). Harden and Young (4) found "no weighable amount of ash" in yeast glycogen prepared without the use of strong alkali. Slater (5), more recently, reported no ash in one sample of glycogen from the mussel, *Mytilus*, and 0.02 per cent in another. Indeed, the absence of ash has been regarded as a criterion of purity by many. On the other hand, Stohmann and Schmidt (6) found 0.045 per cent of ash in a sample of rabbit liver glycogen, prepared by Voit without the use of alkali. More recently, Samec and Isajevic (1) found dog liver glycogen to contain ash even after electrodialysis with ultrafiltration. Meier and Meyerhof (7) purified commercial *Mytilus* glycogen and found over 1 per cent of ash, although the preparations had been dialyzed over 2 hours. A sample of frog muscle glycogen had nearly $1\frac{1}{2}$ per cent of ash. McDowell (8), working in this laboratory, found *Mytilus* glycogen to contain a few hundredths of 1 per cent of ash, even after prolonged electrodialysis with ultrafiltration.

There are then discrepancies on record in regard to the ash content of *Mytilus* glycogen and, by implication, in regard to its phosphorus content. However, the descriptive phrases, "free from ash" or "without weighable amount of ash," are relative terms. A small percentage of ash, such as McDowell found, might be disregarded, especially if the quantity of substance used for the incineration was small. Furthermore, hitherto only two kinds of glycogen have been examined for phosphorus, that from dog liver (Samec and Isajevic) and that from *Mytilus* (McDowell), and of these the liver glycogen contained 0.721 per cent of P_2O_5 and the *Mytilus* glycogen, 0.036 per cent of P_2O_5 . Until more is known concerning the effect of strong alkali in the preparation of glycogen, it is impossible to say whether these differences in ash and phosphoric acid content are specific and indicative of the existence of different glycogens, or whether they are due to the methods of preparation. It seemed, therefore, important in the present investigation to determine also to what extent the ash

content of abalone glycogen may be reduced by electrodialysis and especially to what extent the phosphorus may be removed thereby.

The abalone was chosen as raw material, since in California the flesh of this mollusc may be purchased in butcher shops when in season. These abalone steaks consist of thin slices of the large pedal muscle with which the mollusc adheres to its support and with which it creeps about upon the rocky bottom of the sea coast. As offered in the shops, the steaks have been thoroughly pounded to render them tender. These steaks are perhaps the most readily available local source of glycogen, for Albrecht (9) found their glycogen content high, although he does not seem to have put quantitative determinations on record. The method here reported of preparing abalone glycogen is applicable to other similar materials.

The method itself is nothing other than the method described by Levene nearly 30 years ago for the preparation of nucleic acids (10). 5 pounds of abalone meat, purchased from the iced stock of a local dealer, were minced in a food chopper and then thrown into boiling water and the extraction conducted for about 15 minutes. The liquid was filtered through folded cheese-cloth, the meat then ground to a finer consistency, and again extracted. A third extraction completed the process.

Since the steaks contain an appreciable amount of acid which imparts a very decided acid character to the extraction mixture, the resulting danger of hydrolyzing the glycogen present was minimized by maintaining the mixture neutral to litmus during extraction through the addition of small quantities of NaOH.

To the cold, combined, slightly acidified (with acetic acid) filtrate, totaling about 1.5 liters, was added an excess of aqueous concentrated picric acid solution, thereby precipitating proteins. Picric acid should be added as long as a precipitate forms and a little in excess of that quantity. If insufficient picric acid is added at this stage, filtration is difficult and resulting preparations are not free from substances giving the biuret reaction.

From the filtrate, glycogen is precipitated by the addition, with stirring, of twice its volume of 95 per cent alcohol. After standing overnight, as much as possible of the clear liquid is decanted off and the remainder of the material filtered on a Buchner funnel. It is then washed with 60 per cent alcohol;

but the yellow color of picric acid cannot at this stage be removed entirely by washing. The precipitate, which is usually a grayish tan color, is taken up in the least possible quantity of water and reprecipitated by a double volume of alcohol. This process is repeated four or five times. As the glycogen approaches the pure state, double volumes of alcohol fail to precipitate it without the addition of electrolytes. A small crystal or two of ammonium acetate should then be introduced into the solution.

The glycogen at this stage is free from the picric acid color and, if a sufficient quantity of picric acid has been added, is free from biuret-reacting substances. If it is not, the glycogen must be dissolved in water, picric acid solution again added, the precipitate filtered off, and precipitation with alcohol as above described repeated until the picric acid color has been removed.

The precipitated glycogen is then first washed with 95 per cent alcohol, next with anhydrous ether, and finally dried to constant weight in a vacuum desiccator over CaCl_2 at room temperature. Nearly 2 weeks are required for the product to reach equilibrium. The resulting product is a snow-white powder. However, the physical properties of the preparation are closely connected with the manner in which it is precipitated from solution by alcohol, as well as with the efficiency with which it is washed with alcohol and ether. Lastly, the way in which it is dried is a factor. Too rapid drying, in the early stages particularly, produces a hard, gritty product instead of an extremely light, fluffy one. According to Bizio (11), glycogen dried at atmospheric pressure over CaCl_2 is a half hydrate. In evaluating the ash and phosphoric acid determinations reported below, it should be kept in mind that the preparations were probably not completely anhydrous.

Abalone glycogen, thus prepared, forms opalescent solutions in cold water and gives the usual coloration with iodine. It does not give the Lassaigne test for nitrogen. The yields obtained varied from 8 to 25 gm. per pound of fresh muscle. These variations are due presumably in part to varying richness in glycogen of the original material, but probably even more to varying losses in the process of purification, depending upon the number of reprecipitations. No effort was made to determine the conditions for obtaining maximum yields nor was any attention given to making the method quantitative.

The ash content of abalone glycogen thus prepared varied. Preparation A contained 0.6 per cent of ash and 0.26 per cent of P_2O_5 , as estimated by the method of Embden with, as in all cases, about 1 gm. of substance for each analysis. Preparation B, on the other hand, had an ash content of only 0.069 per cent and a P_2O_5 content of 0.039 per cent. As will be shown below, it was not possible to reduce the ash content much below this value. Preparation B was one in which insufficient picric acid had been added in the first place, for it was found when tested before drying to give the biuret reaction. It was, therefore, again put through the picric acid process and a biuret-free preparation finally obtained. It had, thus, been reprecipitated more frequently than Preparation A, and this presumably accounts for its lower ash content. As recorded by McDowell (8) for *Mytilus* glycogen, inorganic phosphoric acid could not be detected in neutral or slightly acidified solutions by means of Embden's reagent.

The ash is nearly white with a pale bluish green tint, suggesting the presence of copper. The ash of Preparation B was tested for this metal with negative results, although calcium, magnesium, and iron, as well as phosphoric acid, were identified. The ash of Preparation A was not analyzed.

A 10 per cent solution of Preparation A was electrodialed with ultrafiltration several hours in the Bechhold-König apparatus, the direction of the current being changed from time to time. The membranes used in these and all the other experiments were made from 10 per cent acetic acid-collodion and the current was 110 volts d.c. When a constant low reading on the ammeter had been obtained, the glycogen was reprecipitated with the addition of a trace of ammonium acetate, dried, and analyzed. It was found that dialysis had reduced the ash content from 0.6 per cent to 0.065 per cent and the P_2O_5 content from 0.26 per cent to 0.061 per cent. It will be noted that the ash content is but slightly greater than the P_2O_5 content, so that there can be little other than P_2O_5 in the ash. It is possible that in the ash determinations some P_2O_5 may have been volatilized. The P_2O_5 determinations were not made upon the ash; but for this purpose a separate portion of substance was used and the organic material destroyed in the wet way with sulfuric acid.

100 cc. of a 6 per cent solution of Preparation B were next

electrodialyzed with ultrafiltration as above, but without changing the direction of the current. After 7 hours, the ammeter readings having become constant, a sample was withdrawn for analysis. The ash content had only dropped from 0.069 per cent to 0.065 per cent. The total phosphorus had only dropped from 0.039 per cent to 0.037 per cent.

The dialysis was continued for another 7 hours. During the 14 hours of dialysis the ammeter reading fell over a range of less than 10 ma. The dialysis was then concluded and the glycogen again analyzed. The ash content was then 0.065 per cent and the total P_2O_5 , 0.037 per cent. Electrodialysis for 14 hours, therefore, had left the ash and P_2O_5 content practically unchanged. The ash of the dialyzed glycogen was pure white and gave positive tests for iron,¹ calcium, and phosphates.

The experiment was repeated, but instead of the Bechhold-König apparatus one improvised in the laboratory was used. This consisted of a short Pyrex glass tube, about 4 cm. in diameter held horizontally and closed at either end with rubber stoppers. Through holes in the stoppers were inserted two small porcelain balloons bearing the membranes. The platinum wire electrodes were inserted into the balloons which were closed with perforated rubber stoppers, provided with an arrangement to remove the dialysate as fast as formed. The electrodes were about 2 cm. apart. In the upper border of the Pyrex glass tube was a hole through which the liquid undergoing treatment between the two balloons might be sampled or stirred. This apparatus differs from the Bechhold-König set-up in that it permits the liquid undergoing electrodialysis to be observed through the glass tube and in that the current passes horizontally through the liquid instead of vertically. Hence any separation occurring in the liquid may be observed.

In this apparatus a second portion of Preparation B was dialyzed without change of direction of the current. In the course of the

¹ Gatin-Gruzewska (3) attributed the presence of iron in some of her preparations to contamination with the iron contained as an impurity in the KOH used in the process of preparation. This, of course, fails to account for the traces of iron contained in the abalone glycogen preparations. In this connection, it is perhaps significant that Albrecht (9) found a large amount of iron in abalone tissues.

dialysis the liquid separated into an upper water-clear layer, not appreciably opalescent, and a lower denser opalescent layer, in which was no flake formation—simply a deepening of the opalescence. A slight agitation of the apparatus restored the dispersion of the glycogen throughout the liquid and no attempt was made to analyze the layers separately. This curious phenomenon will be discussed at greater length below.

After 7 hours of dialysis in this apparatus, the ash content of the glycogen was 0.057 per cent and the total P_2O_5 , 0.047 per cent. After 7 additional hours of dialysis, the ash content was 0.055 per cent and the total P_2O_5 , 0.040 per cent.

A third portion of Preparation B was then subjected to dialysis in the above apparatus for 14 hours. The ammeter reading at the beginning of the operation was 12 ma. As glycogen eventually decreases the permeability of the membranes, though it could not be detected in the dialysate, the process was interrupted at the end of 14 hours. The membranes were then removed and the balloons cleaned with acetic acid under suction. The cleansing fluid became cloudy. Fresh membranes were then put on the balloons and the glycogen solution subjected to electrodialysis for 17 hours longer. The ammeter reading had then dropped to 4.5 ma. The glycogen contained 0.05 per cent of P_2O_5 ; ash was not determined. The dialysate of the last 17 hours was concentrated and tested for P_2O_5 by Embden's reagent with negative results.

It is clear then that abalone glycogen does contain a small amount of P_2O_5 in a form that cannot be removed by electrodialysis with ultrafiltration. The average of twelve determinations made upon two different preparations dialyzed under a number of different conditions was 0.047 per cent. The lowest value found was 0.036 per cent; the highest, 0.068 per cent. This high value was obtained for a sample relatively high in ash that had been dialyzed only several hours. It is difficult to conceive of the P_2O_5 of abalone glycogen being thus firmly held unless it be an integral part of the molecule. If it is indeed a part of the molecule, then the molecular weight must be huge. It is possible, however, that glycogen like starch consists of two fractions, one containing phosphorus, the other free from it. In that event, the percentage of P_2O_5 found would, by itself, furnish no basis for estimating the molecular weight.

The average P_2O_5 content of 0.047 per cent found is but little greater than that found by McDowell for the edible mussel (*Mytilus*), 0.036 per cent. The difference is small and may not be significant. Since it is so small and since the *Mytilus* glycogen was prepared with hot, concentrated KOH, whereas the abalone glycogen was subjected to no more destructive reagents than hot water and a slight excess of picric acid, the presumption is created that strong, hot KOH does not split off phosphoric acid from glycogen—at least from that of molluscs. The observations made in this investigation do not warrant a decision as to whether or not the two glycogens are identical or different chemical individuals. Besides the small difference in P_2O_5 content, the only other difference between the two glycogens observed was that in equal concentrations abalone glycogen solutions were much more opalescent than those of *Mytilus* glycogen. Differences in the opalescence of solutions of glycogen from different sources have been reported by others. Clautriau (12) found yeast glycogen about one-fourth as opalescent as that from fungi or rabbits. Harden and Young (4) found oyster glycogen 2.5 times as opalescent as yeast glycogen, while rabbit liver glycogen was slightly more opalescent than oyster glycogen. Meier and Meyerhof (7) found *Mytilus* glycogen less opalescent than frog muscle glycogen. They regard it as less aggregated than frog muscle glycogen.

The settling of glycogen during electrodialysis in the apparatus of Pauli (13) has been observed by Samec and Isajevic (1), but their observations are not comparable with those here reported, because they used solutions of dog liver glycogen that had been heated to 120°. They found that such solutions separate into two parts. This is analogous to the behavior of similarly heated starch solutions which these investigators found separated into a lower gel layer of amylopectin, so called, containing about 80 per cent of the starch substance, and an upper layer of amylose, containing most of the rest of the substance. Practically all the P_2O_5 is found in the lower gel layer (14). Glycogen solutions, however, they found separate into a lower layer containing only 20 per cent of the glycogen and only a minor fraction of the P_2O_5 present, and a clear upper layer containing the rest of the glycogen. Samec and Isajevic believe, therefore, that electrodialysis fractionates dog liver glycogen into two distinct components. Samec and Haerdtl

(14) and Samec and Mayer (15) attribute the fractionation of starch by electrodialysis to the fact that amylopectin, being a phosphoric acid ester, is more electronegative than amylose. For the behavior of glycogen in which the gel fraction separated by electrodialysis was found to contain less P_2O_5 than the original substance no explanation is presented by Samec and Isajevic.

The writers, in their experiments in which electrodialysis was conducted with electrodes placed at the same level so that the path of the current was horizontal between them, found the upper strata of the liquid began to clear up soon after the current was turned on and the separation into an upper clear layer and a lower opalescent one became marked in a little while. There was, however, no evidence that the glycogen was thereby divided into two fractions for the water-clear upper layer gave no color whatever with iodine. All the iodine-reacting substance must have descended into the opalescent lower layer. If no current was sent through the electrodes, no separation occurred. The line of demarcation between the two layers was horizontal and ultimately dropped below the level of the balloons. It is not likely that the disappearance of glycogen from the clear layer was due to its removal either by deposition upon one of the membranes or by passage through either of them. As already stated, no notable deposit was found on the membranes nor could more than traces of glycogen be detected in the dialysate. There is undoubtedly a sinking of glycogen into the lower layer, for this becomes manifestly much more opalescent than the original solution. Furthermore, if the whole solution be removed from the apparatus, mixed, brought to its original volume, its opalescence is the same as that of a portion of the solution that was never introduced into the apparatus and never dialyzed, as determined with a nephelometer. There is no sign of flocculation or coagulation, at least of so gross a character as might be detected by such simple means. Moreover, when the dialyzed solution was removed from the apparatus, mixed, and allowed to stand in a tall vessel, as little separation occurred as in undialyzed glycogen solutions.

Svedberg offers the suggestion for this rather frequent behavior of colloids that "The phenomenon is probably due to cataphoresis of the colloid, which makes the solution more concentrated at one

membrane and produces clear liquid at the other. On account of the difference in density the former flows down to the lower part of the vessel and the latter rises to the upper part" (16). The writers have convinced themselves that this is the correct explanation by the following experiment: A septum of mica was introduced midway between the two balloons in such manner that there was free communication through the upper half of the tube but not through the lower half. The glycogen wandered to the anode side of the septum so that the cathode side of the septum became clear and the glycogen as it accumulated on the anode side sank to the bottom on that side. Ultimately, nearly all the glycogen accumulated as a very opalescent lower layer on the anode side of the septum. The liquid in all the rest of the tube, except a thin layer at the bottom of the cathode side, was clear. It is possible that the introduction of a septum in this manner may turn out to be a practical improvement in the technique of concentrating colloids by the electrodialysis method of Pauli, who discovered this phenomenon as exhibited by proteins (13).

As shown above, ordinary opalescent glycogen solutions do not behave in the same manner as the autoclaved solutions of Samec and Isajevic. This is also true for unheated opalescent starch solutions. The writers electrodialyzed opalescent potato and maize starch solutions prepared by suspending dry ground starch in cold water (17) and filtering, and found that the opalescent material settled out in a lower layer beneath a clear upper layer. The clear upper layer could not have contained much amylose, since on addition of iodine it gave a greenish coloration which turned blue only on standing for some time. Even then the blue coloration was not intense. These experiments are not comparable with those of Samec and Haerdtl, since the starch had not been subjected to heat or chemical reagents, whereas Samec and Haerdtl used autoclaved starch. In view of the observations here recorded and in view of the importance of the conclusions drawn by Samec and Haerdtl from their electrodialysis experiments, it would seem that the behavior of untreated starch in electrodialysis requires further investigation.

SUMMARY.

A method is described for the preparation of glycogen without the use of powerful reagents.

Glycogen was prepared by this method from the red abalone, *Haliotis rufescens*, Swainson.

Abalone glycogen was found to contain 0.047 per cent of P_2O_5 (average of twelve analyses) that could not be removed by prolonged electrodialysis with ultrafiltration.

It was found impossible by electrodialysis to obtain abalone glycogen free from ash. The ash of glycogen that had been thoroughly electrodialyzed contained, besides P_2O_5 , very small amounts of calcium and iron.

During electrodialysis of opalescent glycogen solutions, the glycogen sank to the lower part of the containing vessel without visible flocculation.

No evidence was obtained that electrodialysis separates unheated glycogen into two fractions, one poor in or free from P_2O_5 , the other rich in P_2O_5 . No evidence was obtained that abalone glycogen is a mixture of chemical individuals.

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A CHEMICAL STUDY OF CH 'AN SU, THE DRIED VENOM OF THE CHINESE TOAD, WITH SPECIAL REFERENCE TO THE ISOLATION OF EPINEPHRINE.*

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A brief summary of our work on Ch'an Su appeared recently in a preliminary communication (1). A historical account of the toad poison and a pharmacognostic study of Ch'an Su by us have been published elsewhere (2).

The chemical investigation of the secretion of the toad was begun by Pelletier (3), and continued by Gratiolet and Cloëz (4), Fornara (5), Calmels (6), Phisalix and Bertrand (7), Bertrand (8), and Faust (9). The highly potent substances prepared by Phisalix and Bertrand, and by Faust were still amorphous products. Abel in association with Macht (10) was the first to isolate bufagin and epinephrine in crystalline form from the American tropical toad, *Bufo aqua*. Wieland and his coworkers (11) later succeeded in obtaining crystalline bufotalin and bufotoxin from the European common toad, *Bufo vulgaris*. Apparently the European toad does not secrete epinephrine. Handovsky (12) separated from the same species a substance which he thinks is a pyrrole derivative and distinctly different from epinephrine. Novaro (13) claimed that the secretion of *Bufo marinus* contains epinephrine, but he did not isolate epinephrine in chemically pure form.

Ch'an Su has been investigated under the name Senso by several Japanese workers during the last few years. Shimizu (14) isolated from Ch'an Su cholesterol and bufagin, $C_{15}H_{24}O_4$, m.p. 209–210°, which he believed to be identical with Abel's bufagin,

* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

without, however, giving any chemical proof of such identity. He also separated an amorphous substance which he termed bufotoxin and which did not contain nitrogen, and further indicated the probable presence of epinephrine by color reactions and biological tests, but was not able to isolate the latter in chemically pure form. Kodama (15) gave the formula $C_{27}H_{34}O_7$ for bufagin, m.p. $222-223^\circ$, and claimed to have succeeded in obtaining bufotoxin to which he ascribed the formula $C_8H_{10}O_2$, m.p. $203-204^\circ$, in crystalline form. Kotake (16), repeating the work, found the formula for bufagin to be $C_{29}H_{38}O_7$, m.p. $220-221^\circ$, and Kodama's bufotoxin to be a chlorine-containing compound, $C_{27}H_{35}O_5Cl$, m.p. $218-220^\circ$. One can see from the foregoing that the chemical results so far obtained differ widely.

Using the same procedure as has been applied by Abel and Macht in the isolation of epinephrine from *Bufo agua*, we were able to obtain this substance in crystalline form from Ch'an Su as will be seen from the experimental part of this paper, and thus prove the presence of epinephrine in the Chinese product.

Furthermore we have found evidence of the chemical identity of the bufagin which was isolated by us from Ch'an Su with Abel's bufagin. The melting points of both products, purified from the same solvent, were the same and showed no depression when the two substances were mixed. We also prepared acetyl derivatives of both products which had the same melting point and showed no depression in melting point when mixed. We realize only too well that the identity of these two compounds can be made certain only by preparing several derivatives and decomposition products and comparing them. But unfortunately we had only 100 mg. of Abel's bufagin at our disposal. As soon as an opportunity presents itself we hope to be able to settle this question definitely.

Our main aim in taking up the chemical investigation of Ch'an Su was however to see if it also contained a substance which was similar in its chemical composition to the bufotoxin isolated by Wieland and Alles (11) from the European toad, *Bufo vulgaris*. Wieland and Alles were able to show that the molecule of bufotoxin is made up of bufotalin and suberyl-arginine. We have been successful in obtaining from Ch'an Su a nitrogen-containing substance which seems to be closely related in its chemical com-

position to the bufotoxin of Wieland. Our product gives a positive Sakaguchi test (17), thus indicating the presence of arginine in the molecule. We have also isolated from Ch'an Su a fatty acid which seems to be suberic acid. Our finding in Ch'an Su of a compound which is apparently similar in its chemical composition to the bufotoxin of Wieland refutes the claim of the Japanese chemists, Shimizu (14) and Kodama (15), to have isolated bufotoxin from Ch'an Su, as their compounds do not contain nitrogen. As very thorough analytical work is required to establish the empirical formulas of these compounds we shall report later in more detail about this phase of our work on toad poison. Here we shall describe only the isolation of epinephrine from Ch'an Su.

EXPERIMENTAL.

150 gm. of finely powdered Ch'an Su¹ were mixed thoroughly with 400 cc. of 1 per cent acetic acid and let stand for 2 days in the dark. There was formed such a colloidal emulsion, due to the presence of mucin in Ch'an Su, that it was impossible to effect a separation either by filtration or by centrifugation at high speed. After addition of 600 cc. of 96 per cent alcohol, a fairly good separation was obtained by centrifuging the solution at high speed for 15 minutes. The alcoholic water solution, which still had quite an amount of matter in colloidal suspension, was evaporated to about 300 cc. under diminished pressure in a carbon dioxide atmosphere. To the thick and slimy mass about 200 cc. of water were added and then enough basic lead acetate solution until a clear separation of solid matter and solvent was effected. The solution was filtered with suction and the precipitate washed well with 1 per cent acetic acid. The lead was removed from the filtrate with hydrogen sulfide and the filtrate from the lead sulfide evaporated to about 250 cc. under diminished pressure in a carbon dioxide atmosphere. After filtration the solution was shaken out three times with chloroform to remove organic matter like bufagin and other substances. The watery solution was then further evaporated to about 30 cc. with the same precautions.

¹ One will have to protect his nose from the inhalation of the powder, which is very irritating, causing sneezing and hypersecretion.

To the clear dark red solution concentrated ammonia was added until the reaction was strongly alkaline. Petroleum ether was added to the solution to prevent further oxidation and the mixture put in the ice box overnight. The precipitate was filtered off with suction, washed well with dilute ammonia water, then with alcohol and ether. About 350 mg. of a grayish looking crystalline powder were obtained from 150 gm. of Ch'an Su. This powder already possessed a high degree of purity as shown by the melting point and by biological comparison with other samples of epinephrine from *Bufo aqua*, prepared by Abel. The product was purified by dissolving in acetic acid and again precipitating with ammonia. After two precipitations a very pure colorless product, showing the crystalline form of epinephrine, was obtained.

The product melted at 212° with decomposition; when mixed with epinephrine, obtained from *Bufo aqua*, no depression in melting could be observed.

Analysis.—(Micro-Kjeldahl.)

3.663 mg. substance: 2.005 cc. 0.01 N HCl.

3.515 " " : 1.977 " 0.01 " "

$C_9H_{13}NO_3$. Calculated N. 7.65 per cent.

Found. " 7.66 and 7.86 per cent.

The specific rotation of our product was found to be

$$[\alpha]_D^{25} = -49.83^{\circ}$$

This value is in close agreement with that reported for *l*-epinephrine by different investigators.

SUMMARY.

A chemical study of the constituents of the dried Chinese toad poison, Ch'an Su, was made. The blood pressure-raising principle of Ch'an Su was isolated in crystalline form and found to be epinephrine.

We wish to express our appreciation to Professor Abel for supplying us with a sample of epinephrine and bufagin, obtained by him from the toad, *Bufo aqua*.

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CORRECTION.

The statement in the text that the mixed melting point of bufagin from *Bufo agua* and from Ch'an Su and that of their acetyl derivatives showed no depression is incorrect. The mixed melting point of the above named compound shows a marked depression. This suggests that the bufagin from *Bufo agua* is chemically different from the bufagin from Ch'an Su. In a later paper the relationship of these two compounds will be shown.

THE DIGITALIS GLUCOSIDES.

III. GITOXIGENIN AND ISOGITOXIGENIN.

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(Received for publication, April 2, 1929.)

In a previous communication,¹ the conclusion was reached that gitoxigenin is a $\Delta^{6,\gamma}$ -lactone like digitoxigenin² and the related cardiac aglucones. In certain respects, however, it exhibited an abnormal behavior which suggested a structural divergence from the latter. This dissimilarity became evident in the study of derivatives of isogitoxigenin which had been in turn obtained in rather poor yield by the isomerizing effect of alkali on gitoxigenin. Isogitoxigenin resembled the other iso compounds in that it no longer gave the nitroprusside reaction and resisted all attempts at catalytic hydrogenation. Its formation, therefore, must have involved the double bond of gitoxigenin. When saponified, isogitoxigenin yielded isogitoxigeninic acid. Contrary to the other iso acids, this substance did not appear to behave as a hydroxy-aldehyde which may react in either the aldehydic or lactol form. As the methyl ester it did not yield a semicarbazone. When it was oxidized with chromic acid a neutral ester, the so called isogitoxigenonic methyl ester, was formed. Saponification experiments with this substance performed in the usual manner seemed to exclude the formation of a lactone group contrary to the experience with similar derivatives of isodigitoxigenin, etc. Finally, an acid was obtained on oxidation of isogitoxigeninic acid with hypobromite, which proved to be monobasic, and with this also no evidence of the formation of a lactone group was obtained by the customary procedure.

¹ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **79**, 553 (1928).

² Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **78**, 573 (1928).

However, a reexamination of the evidence and further study have caused us to alter the previously published suggestion that degradation occurs during the hypobromite oxidation. The facts now available show definitely that isogitoxigenin, like the other iso compounds, is a lactone on the lactol form of a hydroxyaldehyde. Contrary to our former belief, isogitoxigenin when saponified displays great stability towards alkali. Renewed study has resulted in a great improvement in the yield of the iso compound which can be obtained from gitoxigenin. Reinvestigation of the preparation and composition of the so called isogitoxigenonic methyl ester mentioned above has confirmed the previously reported formulation, $C_{24}H_{34}O_6$. But its relationship to the parent substance was misinterpreted. This ester had been found to consume only 1 equivalent of 0.1 N alkali when saponified by the method which opened both ester and lactone group in the case of the analogous isodigitoxigenin isostrophanthidin,³ etc., derivatives. However, more recently, by the use of stronger alkali and higher temperature, it has become possible to detect a relatively resistant lactone group in the isogitoxigenin derivative. This substance is, therefore, a ketolactone ester and in conformity with the analogous substances obtained from the other iso compounds should be called *isogitoxigenonic methyl ester*.

More recently a similar experience has been encountered with the hypobromite oxidation product mentioned above. From the former analytical experience with this substance, the incorrect formula $C_{21}H_{30}O_6$, was derived. A renewed study of the preparation and purification of this acid has resulted in an unquestionably pure, anhydrous substance. The analytical figures obtained are now in agreement with the normal formulation $C_{23}H_{34}O_6$. As previously described, this substance neutralizes 1 equivalent of alkali on direct titration and when boiled with an excess of 0.1 N alkali extra consumption of the latter was negligible in contradistinction to the experience with similar derivatives of other iso compounds. However, with stronger alkali and higher tempera-

³ Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **61**, 395 (1924). Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 531 (1928). Jacobs, W. A., and Heidelberg, M., *J. Biol. Chem.*, **81**, 779 (1929).

ture an extra equivalent was consumed. This substance, *isogitoxigenic acid*, is therefore a lactone acid which is isomeric with isoperiplogenic acid⁴ and isosarmentogenic acid.⁵ It differs from the latter substances in the greater stability of its lactone group.

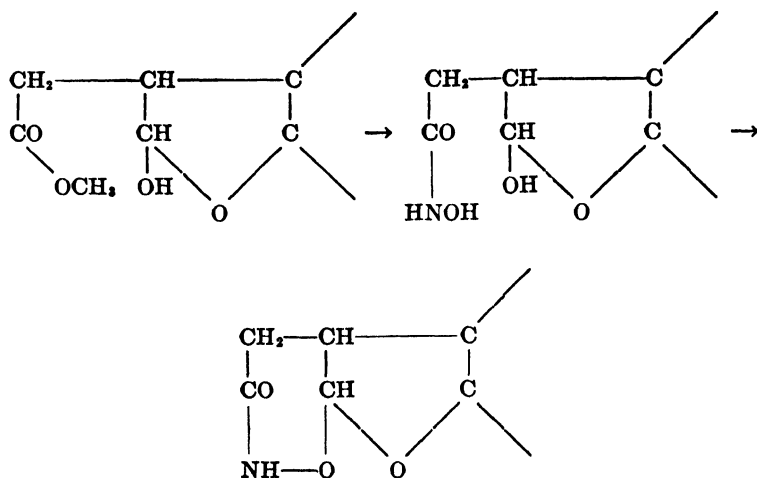
Gitoxigenin like digitoxigenin and the other well studied cardiac aglucones possesses one secondary alcoholic group and a tertiary hydroxyl which is presumably γ to the aldehydic carbon. The retention of the secondary hydroxyl in isogitoxigenin has been shown by its oxidation to the ketone *isogitoxigenon*. If one assumes the probable similarity in other respects of the general structural make-up of both gitoxigenin and digitoxigenin, it is possible that the difference in chemical properties noted above may be attributable to the position occupied by the extra tertiary hydroxyl of gitoxigenin. If this were so, the isogitoxigenin derivatives should present a normal behavior after removal of this hydroxyl. This suggestion was supported by the following observation.

Isogitoxigenic acid was readily converted by strong hydrochloric acid into *anhydroisogitoxigenic acid*, due to the removal of the additional tertiary hydroxyl as water. This anhydro acid now presented the normal behavior in that its lactone group was much more readily opened by dilute alkali. It appears probable that the proximity of the extra tertiary hydroxyl group plays a rôle in the stability of the lactone group of isogitoxigenic acid. Probably, for similar reasons isogitoxigeninic acid exists only as the stable lactol and not as a hydroxyaldehyde. The previously reported failure of its ester to yield a semicarbazone would thus be explained. This has its parallel in our experience with pseudostrophanthidin⁶ which was shown to be a γ -lactol. However, to submit isogitoxigeninic methyl ester to a further test, we have attempted its reaction with hydroxylamine. A reaction occurred, but instead of an oxime a substance was obtained which owed its origin apparently to the intermediate formation of a hydroxamic acid which then lost water with the lactol hydroxyl as shown in the following scheme:

⁴ Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, **79**, 529 (1928).

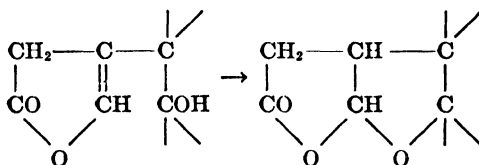
⁵ Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, **81**, 778 (1929).

⁶ Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **65**, 495 (1925).



This substance gives a deep red purple color with ferric chloride which is characteristic of hydroxamic acids.

The present observations permit the definite conclusion that gitoxigenin like digitoxigenin is a tetracyclic $\Delta^{\beta,\gamma}$ -lactone in which a carbon atom presumably γ to the lactone γ carbon atom carries a tertiary hydroxyl group. In the formation of isogitoxigenin this hydroxyl functions in an oxidic union between the two carbon atoms with a disappearance of the double bond as follows:



Additional work will shortly be presented on attempts at further structural correlation of gitoxigenin with digitoxigenin by means of the iso compounds.

We are especially indebted to both Merck and Company, Inc., of Rahway and E. Merck, Darmstadt for their generous gift of the "insoluble digitoxin by-product" which was the source of the gitoxigenin used in this investigation.

EXPERIMENTAL.

Isogitoxigenin.—The following improvement in the method for the isomerization of gitoxigenin was developed, which departed from that previously employed by the use of stronger reagent.

1 gm. of isogitoxigenin was shaken at 25° in a solution of 1 gm. of potassium hydroxide in 10 cc. of dry methyl alcohol. Solution gradually occurred and at the end of 15 minutes the solution was well diluted. Acidification to Congo red caused an immediate precipitate which gradually crystallized. After standing 24 hours to insure complete relactonization, the mixture was repeatedly extracted with chloroform. After washing with dilute carbonate, the dried extract yielded on concentration a crystalline residue which was collected with a few cc. of dry chloroform and ether. The total yield was 0.70 gm. When recrystallized from alcohol, it softened above 220° and finally melted at 252°. In all other properties, it agreed with those already recorded. When heated in a sealed tube at 130° for 4 hours in an excess of *N* sodium hydroxide solution, unchanged isogitoxigenin was recovered after acidification to Congo red and relactonization.

Isogitoxigenic Methyl Ester.—Isogitoxigeninic methyl ester was oxidized as previously given with the slight modification that a mixture of 4 parts of acetic acid and 1 part of water was used as the solvent. When recrystallized by the cautious addition of dry ether to its concentrated methyl alcoholic solution, the ketolactone ester slowly separated as broad, flat prisms which melted sharply at 174°.

5.135 mg. substance:	3.815 mg. H ₂ O,	12.970 mg. CO ₂ .
5.006 "	3.770 "	12.614 "
	C ₂₄ H ₃₄ O ₆ . Calculated.	C 68.86, H 8.19.
	Found.	" 68.87, " 8.32.
		" 68.72, " 8.42.

That the substance is the ester of a lactone acid was shown as follows: 0.98794 gm. of substance was heated at 125–130° for 5 hours in a sealed tube with 1 cc. of alcohol and 2.190 cc. of *N* NaOH in an atmosphere of nitrogen and then titrated back against phenolphthalein. Found 0.412 cc. Calculated for 2 equivalents, 0.472 cc.

Isogitoxigenic Acid.—Isogitoxigeninic acid was oxidized with hypobromite as previously described. The acid, however, was

obtained from the reaction mixture by acidification with acetic acid instead of sulfuric acid. On rubbing, the clear solution deposited the crystalline lactone acid. For recrystallization the substance was dissolved in 50 per cent alcohol by the addition of ammonia. Reacidification with acetic acid after warming and careful dilution caused the acid to separate as microrosettes of leaflets which were anhydrous and melted at 260° .

$$[\alpha]_D^{25} = -50^{\circ} \text{ (c = 0.993 in 95 per cent alcohol.)}$$

3.404 mg. substance: 2.622 mg. H_2O , 8.460 mg. CO_2 .

3.333 " " : 2.560 " " 8.288 " "

$C_{23}H_{34}O_6$. Calculated. C 67.99, H 8.44.

Found. " 67.78, " 8.62.

" 67.81, " 8.60.

The lactone group of this acid was only incompletely saponified by boiling for 4 hours in N alkali. Correct results were obtained as follows: 0.10063 gm. of substance was sealed in a tube with 2 cc. of alcohol and 5.425 cc. of N NaOH in an atmosphere of nitrogen. After heating at 125 – 130° for 5 hours the mixture was titrated back against phenolphthalein. Found 0.480 cc. of N NaOH. Calculated for 2 equivalents, 0.495 cc.

To complete the data a direct titration was made with 0.1 N NaOH. 17.480 mg. of substance in 2 cc. of alcohol required 0.425 cc. of 0.1 N NaOH. Calculated for 1 equivalent, 0.430 cc.

Anhydroisogitoxigenic Acid.—0.1 gm. of isogitoxigenic acid was dissolved at 20° in 5 cc. of hydrochloric acid (1.19). After about 10 minutes crystallization readily occurred on rubbing. After several hours the substance was collected with hydrochloric acid followed by water.⁷ The acid was recrystallized by dissolving a suspension in 50 per cent alcohol with the aid of ammonia and reacidification with acetic acid. It formed on standing aggregates of platelets which were anhydrous and melted at 215° after preliminary softening.

4.713 mg. substance: 3.500 mg. H_2O , 12.267 mg. CO_2 .

$C_{23}H_{32}O_6$. Calculated. C 71.08, H 8.31.

Found. " 70.97, " 8.31.

⁷ After this paper was sent to press it was found that this substance as directly obtained contains chemically bound chlorine and that during the recrystallization with the aid of ammonia the halogen has been removed with the formation of the anhydro acid described above. The nature of this intermediate chloro compound is under investigation.

Contrary to the parent substance, the anhydro acid was readily saponified by 0.1 N sodium hydroxide.

13.340 mg. of substance were dissolved in 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found 0.347 cc. Calculated for 1 equivalent, 0.344 cc.

2.6 cc. of 0.1 N NaOH were added to the above titration mixture which was then refluxed for 4 hours in an atmosphere of nitrogen and then titrated back. Found 0.341 cc. Calculated for 1 equivalent, 0.344 cc.

Isogitoxigeninic Methyl Ester and Hydroxylamine.—0.1 gm. of isogitoxigeninic methyl ester was refluxed with a solution made by mixing 0.1 gm. of hydroxylamine hydrochloride and 0.2 gm. of potassium acetate in 10 cc. of methyl alcohol and filtering. After 6 hours a crystalline substance was obtained after dilution. On concentration of its solution in methyl alcohol the substance formed aggregates of minute platelets which were anhydrous. It melted with decomposition at 287°. The alcoholic solution gave a deep purple red color with ferric chloride. The substance was neutral and contained no methoxyl.

3 730 mg. substance: 2.947 mg. H_2O , 9.285 mg. CO_2 .

6.050 " " : 0.191 cc. N (24°, 752.5 mm.).

$C_{23}H_{40}O_6N$. Calculated. C 68.10, H 8.75, N 3.45.

Found. " 67.88, " 8.84.

N 3.59.

Isogitoxigenon.—A solution of 0.2 gm. of isogitoxigenin in a mixture of 4 cc. of acetic acid and 1 cc. of water was treated with an excess of Kiliani chromic acid solution, which caused a prompt reaction. After dilution, crystallization was induced by the addition of saturated ammonium sulfate solution. The ketone slowly separated from its concentrated alcoholic solution as sheaves of delicate needles which melted at 278–279° and were solvent-free. It proved to be easily soluble in chloroform, acetone, and less readily so in methyl and ethyl alcohol. It was but sparingly soluble in ether.

3.280 mg. substance: 2.496 mg. H_2O , 8.578 mg. CO_2 .

$C_{23}H_{42}O_6$. Calculated. C 71.08, H 8.31.

Found. " 71.30, " 8.50.

A NOTE ON THE VAN SLYKE METHOD FOR THE DETERMINATION OF CHLORIDES IN BLOOD AND TISSUE.

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Wilson and Ball (1928) have suggested a modification of the Van Slyke (1923-24) chloride method. This consists essentially of adding silver nitrate and nitric acid separately, so that the chloride and proteins are not precipitated simultaneously. Wilson and Ball apparently obtained low chloride values in some samples of dog and human sera when using the standard Van Slyke procedure.

Since much work on blood and serum chlorides had been done in this laboratory, it was decided to compare Wilson and Ball's method with those employed here to assure ourselves that the latter were trustworthy and that our analytical data could be reported without danger of criticism. The results of these studies have demonstrated that the Van Slyke method as employed in the laboratory has proved entirely accurate.

When the Van Slyke method was introduced into this laboratory, numerous control studies indicated that it was entirely satisfactory. Added chloride was uniformly quantitatively recovered. As a matter of accident or convenience, the method was always used under the same conditions. The nitric acid-silver nitrate solution was added to the blood in a large Pyrex test-tube, with constant stirring to insure quick and complete mixture of the two fluids. The contents of the tubes were then digested on a steam bath, overnight in the case of serum, and 24 hours in the case of whole blood. Small inverted volumetric flasks were used as condensers instead of the watch-glasses prescribed by Van Slyke. The mixture was cooled, usually in running water, so that it was never above room temperature when it was titrated.

Fiske and Sokhey (1925) used potassium permanganate to facilitate digestion in the von Korányi method (1897) for serum chlorides. Later permanganate was employed here in the Van Slyke method for the same purpose. Concentrated potassium permanganate solution is added to the boiling digestion mixture until a pale yellow solution results. Then water (about 5 cc.) is added, the solution boiled again, and several more drops of potassium permanganate solution are added. Cooling and titration are carried out as in the regular Van Slyke method. This modification was adopted only because it is possible by means of it to obtain chloride results within 15 minutes instead of on the following morning.

Results on whole blood, serum, and stool from this more vigorous form of digestion were always the same as those from the regular Van Slyke method. Although it seemed reasonably certain from these studies that the Van Slyke method with or without permanganate was accurate and satisfactory, nevertheless it seemed advisable to compare these techniques with Wilson and Ball's modification.

EXPERIMENTAL.

Seven samples of human blood and eleven samples of serum were analyzed for chloride by the several methods: (1) Wilson and Ball method (1928); (2) Van Slyke method (1923-24); (3) Van Slyke method plus permanganate; (4) Wilson and Ball method with its essential requirement of adding silver nitrate and nitric acid separately retained, while potassium permanganate solution was added to assist digestion; (5) Whitehorn method (1920-21).

Results.

The data are to be found in Table I. The error of any one method is about 0.8 milli-equivalents. It will be seen that the differences obtained with any of the three to five methods employed rarely exceed this error. Evidently the Van Slyke method with or without permanganate is as reliable as that of Wilson and Ball.

In view of these data, it is difficult to explain the low results obtained by Wilson and Ball, unless our long time digestion and

TABLE I.
Chloride in Blood in Serum.

Method.	Chloride in milli-equivalents in:													
	Blood.							Serum.						
Wilson plus permanganate.	90.0	79.2	74.8	80.4	81.2	80.8	80.4	107.4	111.6	108.4	102.0	100.4	102.4	99.6
	91.6	79.2	75.2	81.2	80.4	80.4	80.4	107.4			101.2	100.8	102.0	99.2
Van Slyke plus permanganate.	91.2	79.2	75.2	81.2	80.6	80.8	80.0	107.6	112.8	108.0	102.4	100.4	102.4	99.0
	90.8	78.8	75.2	80.8	81.4	80.4	80.8				102.4	101.2	102.8	99.2
Wilson.	90.4	78.8	74.8	81.2	81.2	79.6	80.6	106.8	112.4	107.6	101.2	100.8	102.8	99.6
	90.8	79.2	75.6		80.4	79.6	81.0	106.8			102.0	100.4	102.8	99.8
Van Slyke.	90.8	79.2	75.4		80.4	80.8	80.4	107.6		107.6	102.0	101.2	102.8	99.4
		78.4	75.6		81.2	80.4	80.4	107.6			102.0	101.2	102.4	98.8
Whitehorn.		78.8				81.6	81.2							
		78.4								101.6			98.8	

efficient condensation account for the more uniform reliability of the results obtained.

SUMMARY.

Although Wilson and Ball claim that the Van Slyke chloride method gives low results in some human and dog sera, we were unable, in a series of seven bloods and eleven sera (human), to obtain any higher results by the Wilson and Ball method than by the Van Slyke method. It therefore seems justifiable to assume that the Van Slyke method for the determination of chlorides is accurate and reliable.

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BENZOYLATED AMINO ACIDS IN THE ANIMAL ORGANISM.

IV. A METHOD FOR THE INVESTIGATION OF THE ORIGIN OF GLYCINE.*

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(Received for publication, March 14, 1929.)

The results of numerous investigations have indicated that the animal organism has a readily available supply of the amino acid, glycine, which may be used for the detoxication of benzoic acid. These studies, which have been reviewed elsewhere (1), have dealt with the toxicity of benzoic acid, with the effect of the administration of benzoate on the distribution of nitrogen in the urine, and with the extent and rate of formation of benzoyl glycine, or hippuric acid. Although neither these nor perfusion experiments have satisfactorily demonstrated its source, it has been generally assumed that glycine may be synthesized by animals as well as by plants.

Glycine is a component of animal tissues (2, 3) and must be supplied during the period of growth, either in the diet or by synthesis. If the supply in either case were limited, the growth of young animals would be affected by the administration of sodium benzoate, since glycine would then be required for the detoxication of benzoic acid as well as for the synthesis of tissue proteins. For this reason it appeared that the rate of growth of young animals on diets which contained sodium benzoate and which were either rich or poor in glycine or precursors of glycine might throw new light

* Preliminary reports of this work were presented before the American Society of Biological Chemists at Rochester, April, 1927 (Griffith, W. H., *J. Biol. Chem.*, **74**, p. lxxv (1927)) and before the Society for Experimental Biology and Medicine, St. Louis, 1927 (Griffith, W. H., *Proc. Soc. Exp. Biol. and Med.*, **24**, 717 (1927)). •

on the obscure origin of this amino acid. In this paper a study has been made, therefore, of the extent of formation of hippuric acid in rats and of the growth of rats on diets containing varying amounts of sodium benzoate.

EXPERIMENTAL.

Male white rats, 4 to 5 weeks of age and weighing 45 to 60 gm., were used in these experiments. They were kept in raised individual cages with screen bottoms so that neither shavings nor feces were accessible. Weighings were made at 10 day intervals. At the end of the experimental period, usually 40 days, an 18 to 24 hour urine sample was collected and analyzed for total benzoic acid (4), free benzoic acid (5), and hippuric acid (6). Analyses were always

TABLE I.
Composition of Diets.

Diet No.	A	1.5 A*	2 A	2.5 A	3 A	3.25 A	3.5 A	3.75 A	Calo- ries.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
Sodium benzoate...	0.00	1.50	2.00	2.50	3.00	3.25	3.50	3.75	
Casein†.....	35.00	35.00	35.00	35.00	35.00	35.00	35.00	35.00	112
Salt mixture‡.....	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	
Sucrose.....	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	12
Cod liver oil.....	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	36
Corn-starch.....	36.40	33.70	32.80	31.90	31.00	30.55	30.10	29.65	304
Lard.....	17.60	18.80	19.20	19.60	20.00	20.20	20.40	20.60	

* Throughout this paper the number before the letter A refers to the per cent of sodium benzoate in the diet.

† Commercial casein (12.75 per cent nitrogen) Calories calculated on the basis that the casein was 80 per cent protein.

‡ Osborne and Mendel (7).

made on the combined urines of a group of rats on the same diet. In a few instances urines were collected during the experimental period. The urines were preserved with 4 per cent sulfuric acid during collection.

The composition of the experimental diets is given in Table I. Diet A was chosen as the basal diet because sodium benzoate could be conveniently incorporated into it. Diets similar in composition have been rather generally used as control basal diets. This food

mixture also had the advantage of being low in glycine. In every case the diet was supplemented daily with 150 mg. of dried brewers' yeast.¹ Glycine or gelatin, when added to these diets, always replaced an equal weight of starch.

TABLE II.

Excretion of Free Benzoic Acid, Hippuric Acid, and Total Benzoic Acid on Diets Containing Sodium Benzoate.

Diet No.	No. of rats.	Total benzoic acid.	Hippuric acid calculated as benzoic acid.		Free benzoic acid.	Day of urine collection.
		mg.	mg.	per cent of total benzoic acid	mg.	
3.5 A	4	525	385	73	25	35th
		715	516	72	13	53rd
3 A	4	634	449	71	18	41st
	6	516	378	73	16	41st
	5	755	573	76	74	41st
	4	165	117	71	7	41st
	4	570	452	79	8	31st
2.75 A	4	570	452	79	8	31st
2.5 A	5	447	295	66	16	41st
	8	465	372	80	13	41st
	4	352	233	67	4	20th
	6	698	614	88	13	55th
2.25 A	4	570	452	79	8	30th
2 A	6	368	333	90	6	41st
	4	296	244	82	4	41st
	4	832	700	84	0	41st
	6	280	261	94	4	41st
1.5 A	5	217	206	95	2	41st
	13	1430	1370	96	18	41st
3 A + 0.39 per cent glycine.	4	810	720	89	13	10th
3 A + 0.78 " " "	9	975	947	97	6	41st
3 A + 1.04 " " "	4	623	540	86	25	10th
3 A + 1.56 " " "	13	1198	1182	99	12	41st

Only traces of hippuric acid were found in the urines of rats fed the basal diet. After the addition of sodium benzoate to the diet, hippuric acid was readily isolated from the urine and identified by its nitrogen content and melting point. The results of the urine

¹ We are indebted to Mr. O. F. Steidemann of the Laboratory Department of Anheuser-Busch, Inc., for the yeast used in these experiments.

analyses showed that 66 to 95 per cent of the total benzoic acid eliminated in the urine of rats after the ingestion of sodium benzoate was in the form of hippuric acid (Table II). With Diet 1.5 A the synthesis of hippuric acid was nearly quantitative. As the concentration of sodium benzoate in the diet increased, the ratio of hippuric acid to total benzoic acid in the urine decreased. The addition of glycine to Diet 3 A lessened the toxicity of the benzoate

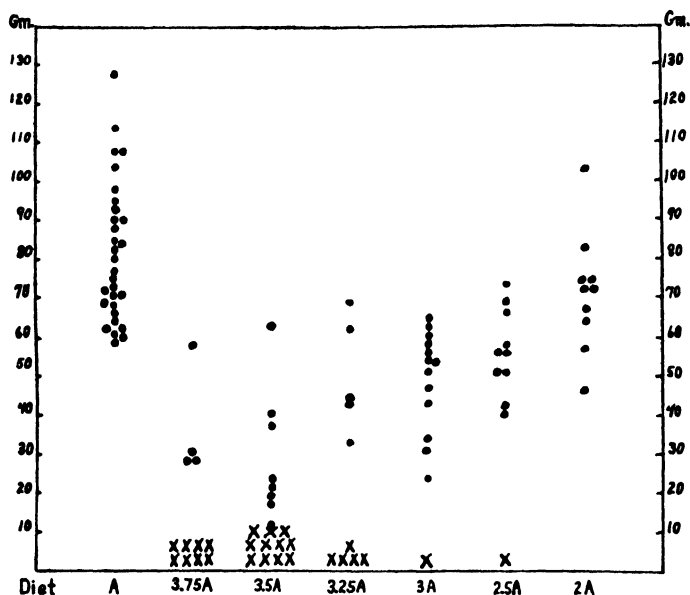


CHART I. The increase in weight of young male rats during a 40 day experimental period on diets containing sodium benzoate. The per cent of sodium benzoate added to the basal diet, Diet A, is given by the number preceding the A. Diets were fed *ad libitum*. Deaths during the experimental period are shown by the letter X.

and increased the proportion of hippuric acid in the urine. Free benzoic acid was seldom found in significant amounts. Most of these urines contained some form of combined benzoic acid other than benzoyl glycine. It is possible that this was benzoyl glycuronic acid since a reducing substance was present. Benzoyl glycuronic acid has been found in the urine of the pig, rabbit, dog, sheep, and man after the administration of benzoate.

The average growth of rats on Diet A during a 40 day experimental period was somewhat less than that of control animals fed a mixed diet and having access to feces and shavings. However, the difference was not marked and Diet A was considered a suitable basal diet. Chart I shows the effect on growth of the addition of sodium benzoate to the basal diet. When over 3 per cent of

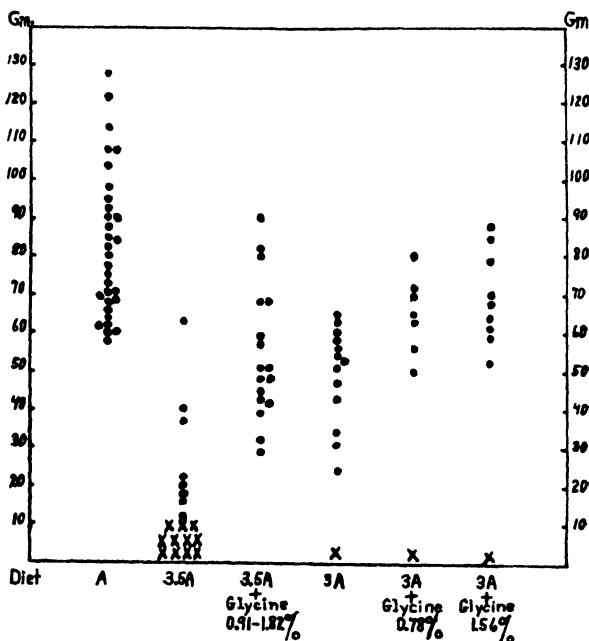


CHART II. The effect of glycine on the increase in weight of young male rats during a 40 day experimental period on diets containing sodium benzoate. The per cent of sodium benzoate added to the basal diet, Diet A, is given by the number preceding the A. Diets were fed *ad libitum*. Deaths during the experimental period are shown by the letter X. The detoxication of 1.0 gm. of sodium benzoate requires 0.52 gm. of glycine.

sodium benzoate was added to this diet, less than one-half of the rats survived and the survivors failed to grow normally. With Diet 3 A, thirteen out of fourteen rats survived but the average weight increment was only 49 gm., approximately 60 per cent of that of the rats on the basal diet (80 gm.). Better growth occurred with Diets 2.5 A and 2 A but even with the latter the rate

of growth was slightly less than that of rats on the basal diet. In a number of cases the experimental period was increased to 100 days but no advantage was found in the longer period.

In these experiments the death of the rats or the failure of the survivors to grow normally was due to the toxicity of the sodium benzoate added to the diet. Detoxication of benzoic acid was inadequate because available glycine was lacking and not because there was inability to synthesize hippuric acid from benzoic acid and glycine. This was evident from the results obtained by adding glycine to the benzoate diets (Chart II). The presence of 0.78 per cent, or more, of glycine in Diet 3 A resulted in a distinct increase in the rate of growth compared to the growth on Diet 3 A alone. This improvement in the rate of growth was associated with an increased extent of synthesis of hippuric acid (Table II). The effect of glycine was more strikingly shown by the results with Diet 3.5 A. The death of eleven out of nineteen rats and the decreased rate of growth of the survivors was evidence of the toxicity of this diet. These results were in marked contrast to those found when glycine was added to the same diet. In a group of seventeen rats there were no fatalities and the weight increment ranged from 29 to 90 gm. The quantity of glycine used in these experiments was that theoretically required for the detoxication of all or part of the benzoate in the diet. In a few cases gelatin, which is 25.5 per cent glycine (8), was used in place of the free amino acid.

The death of rats in these experiments was usually preceded by certain characteristic signs of the toxicity of the benzoate. The animals became restless and irritable and frequently lost the ability to coordinate their movements. Some showed constant tremors involving the whole body, others were subject to convulsive seizures in which the body became rigid. Inflammation of the eyes was common. This was accompanied by an exudate and a thickening of the lid so that often the eyes were completely closed. Diarrhea seldom resulted from the ingestion of these toxic diets.

In a large number of preliminary experiments rats were placed in cages in groups of four rather than in individual cages. The results with Diets 2A and 2.5A were similar to those shown in Chart I. However, with Diet 3A survival was rare. In most cases rats fed this diet became very vicious and fighting occurred at frequent intervals. Although the toxicity of Diet 3 A was greatly

increased under these conditions, the addition of glycine or of gelatin to the diet resulted in survival and varying degrees of growth.

In the experiments reported in Charts I and II it was recognized that the food consumption of rats fed the basal diet *ad libitum* was greater than that of rats fed the diets containing benzoate. It was necessary, therefore, to determine whether the sodium benzoate affected the rate of growth by a specific toxic action other than that of merely decreasing the appetite and food intake. The

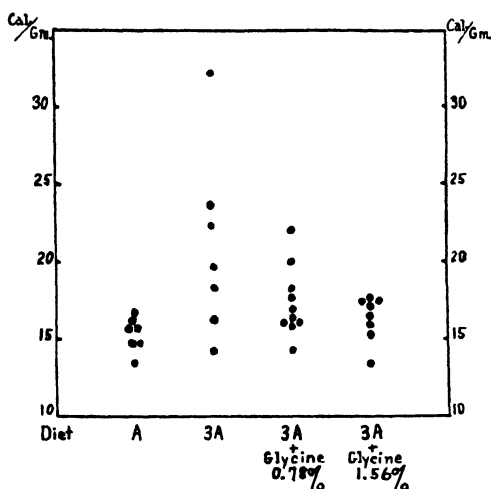


CHART III. The effect of benzoate and of added glycine on the calorific requirement per gm. increase in weight of rats during a 40 day experimental period. The detoxication of 1.0 gm. of sodium benzoate requires 0.52 gm. of glycine.

determination of the food consumption of rats fed *ad libitum* indicated that the requirement of food per gm. of increase in weight was greater on the benzoate diet than on the control diet. This is illustrated in Chart III in which a comparison was made of the calorific requirement per gm. of increase in weight of rats fed Diets A, 3 A, and 3 A plus 0.78 per cent and 1.56 per cent glycine. It was evident that the utilization of food was adversely affected by benzoate and that this adverse effect practically disappeared when glycine was added to the diet.

Although the experiments cited above demonstrated an inhibitory effect of benzoate on growth in addition to the effect on appetite, it was considered advisable to perform a second series of experiments in which all of the rats consumed equal amounts of food of equivalent calorific value. The quantity of food chosen for these experiments was 210 gm., a value only slightly less than the average food consumption (222 gm.) of rats fed Diet 3 A *ad libitum* during the 40 day experimental period. The food was fed as follows: 4.5 gm. daily for the first 10 days, 5.0 gm. daily for the second 10 days, 5.5 gm. daily for the third 10 days, and 6.0 gm. daily for the last 10 days.

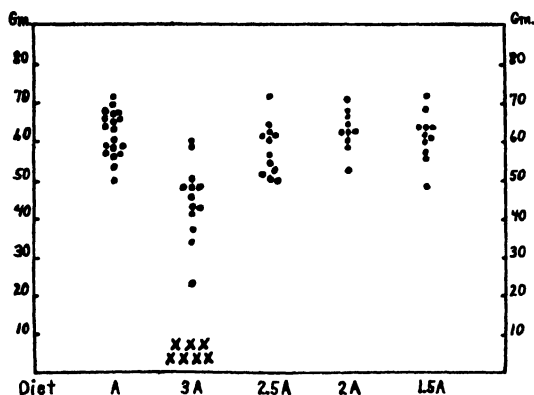


CHART IV. The increase in weight of young male rats during a 40 day experimental period on diets containing sodium benzoate. The food intake was restricted, each rat receiving the same quantity of food of equivalent calorific value. Deaths during the experimental period are shown by the letter X.

Chart IV shows the results of this restricted feeding of Diets A, 1.5 A, 2 A, 2.5 A, and 3 A. As in the first series of experiments the average weight increment of the rats on Diet 3 A (45 gm.) was definitely less than that of the rats on the basal diet (62 gm.), even though the food intake was the same. In addition to this definite inhibitory influence on growth, the proportion of deaths was larger than that found in the first series. This was probably due to the fact that these rats were not as well nourished since their food consumption was somewhat less than that of the rats fed the same diet *ad libitum*.

The average increase in weight of rats on Diets 1.5 A and 2 A, 62 gm. and 63 gm. respectively, was the same as that of the rats on the basal diet (62 gm.). This indicated that glycine was available for adequate detoxication of the benzoic acid in these diets. The beneficial effect of added glycine was very evident in this series of experiments (Chart V). As little as 0.39 per cent of added glycine, an amount theoretically required for the detoxication of only one-fourth of the benzoate of Diet 3 A, was sufficient to permit ten out of thirteen rats to grow at a rate which was comparable to the

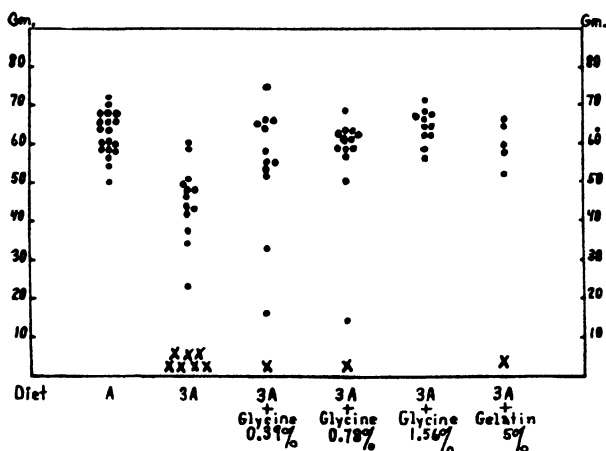


CHART V. The effect of glycine and of gelatin on the increase in weight of young male rats during a 40 day experimental period on diets containing sodium benzoate. The food intake was restricted, each rat receiving the same quantity of food of equivalent calorific value. Deaths during the experimental period are shown by the letter X. The detoxication of 1.0 gm. of sodium benzoate requires 0.52 gm. of glycine.

growth on the basal diet. A similar result was obtained when gelatin, instead of glycine, was added to Diet 3 A.

DISCUSSION.

In these experiments on young growing rats hippuric acid was found to be the most important product in the detoxication of benzoic acid. With a relatively large amount (3 per cent) of sodium benzoate in the diet, 66 per cent, or more, of the ingested benzoic acid was eliminated in the urine as hippuric acid. The

formation of this detoxication product was more nearly quantitative when lower concentrations of benzoate were present in the diet. Practically all of the benzoate ingested by the rats was excreted in a combined form, benzoyl glycuronic acid probably being present in addition to hippuric acid. When the concentration of benzoate in the diet was over 3 per cent, less than one-half of the rats survived. Such diets were toxic because the supply of glycine was inadequate. This was evident from the fact that the addition of glycine to the diet resulted in survival and growth.

The basal diet used in these experiments was a fairly satisfactory source of the preformed glycine or of the precursors of glycine required by the addition to the diet of 2 per cent of sodium benzoate. This was not only true for the experimental period of 40 days but also for periods as long as 100 days. It was concluded, therefore, that glycine, or a precursor, was supplied in the diet and not by the destruction of the body tissues since growth continued during the longer experimental period.

The basal diet was not a satisfactory source of glycine if it contained 3 per cent of benzoate, the rate of growth of the rats being definitely decreased on this diet. In this case glycine was not available in sufficient amounts for the formation of new tissue proteins and for the detoxication of benzoic acid. However, the presence of a relatively small quantity of added glycine was effective in reducing or preventing entirely the injurious effects of the high benzoate diet. The rate of growth of young rats on a diet containing benzoate can be used, therefore, in detecting the presence of glycine or of precursors of glycine in the diet. This method is being used in the study of the origin of glycine in the animal organism. The possibility of developing a similar procedure for the quantitative determination of glycine in proteins and in tissues is being investigated.

The data presented in this paper support the idea that glycine is synthesized by animal tissues but do not furnish any information regarding the nature of the precursors of glycine. In the second series of experiments rats on Diet 3 A ingested 6.3 gm. of sodium benzoate during the 40 day experimental period and excreted 70 per cent of this benzoate as benzoyl glycine. For the formation of this benzoyl glycine 2.3 gm. of glycine were required. According to the analyses of Abderhalden, Gigon, and Strauss (2), glycine

makes up approximately 3 per cent of the total protein of the body, so that the rats at the beginning of the experiment contained from 0.2 to 0.3 gm. of glycine. The diet during the experimental period furnished 73.5 gm. of commercial casein (80 per cent protein) and 6 gm. of dried brewers' yeast (50 per cent protein). Foreman (9) found 0.45 per cent of glycine in casein and Meisenheimer (10) reported that glycine was present in yeast. The diet, then, furnished from 0.3 to 0.5 gm. of glycine. On the basis of the above analyses, it is obvious that the glycine excreted as benzoyl glycine in these experiments could not have come entirely from preformed glycine in the diet or in the tissue proteins. It must be remembered, however, that no quantitative method for the isolation or determination of glycine in protein hydrolysates is available, and it is possible that casein, for example, may contain more than 0.45 per cent of glycine. Foreman, in fact, stated that this value was probably too low. The presence of 4 per cent of glycine in casein would be necessary if synthesis of glycine did not occur and if this protein were the sole source of the glycine available in these experiments.

It has been suggested that glycine may be formed from α -amino- β -hydroxy acids by further oxidation of the β -carbon atom (11). Since casein contains 10.5 per cent of β -hydroxyglutamic acid (12), 7.7 gm. of this amino acid were present in the casein ingested by rats fed Diet 3 A in the second series of experiments. Theoretically, this amount might furnish 3.54 gm. of glycine, 50 per cent more than that supplied by the rats (2.3 gm.). Actually such a source of glycine was not evident for the supply of this amino acid was inadequate for the complete detoxication of the benzoate in Diet 3 A. Inasmuch as the toxicity of this diet could be prevented by a small amount of added glycine, it was not considered probable that glycine was formed from β -hydroxyglutamic acid. It is possible, however, that the benzoate of the diet may have been absorbed from the alimentary tract and may have exerted its toxic action before the products of the digestion of protein were available for detoxication.

Wiechowski (13), McCollum and Hoagland (14), and Lewis (15, 16) reported that, after benzoate administration, the increase in the hippuric acid (glycine) nitrogen was accompanied by a corresponding decrease in the urea nitrogen of the urine and suggested

that glycine was formed from some nitrogenous substance whose nitrogen was normally eliminated as urea. In the second series of experiments reported in this paper, rats on Diet 3 A ingested 10 gm. of protein nitrogen during the experimental period. Of this amount of nitrogen only 0.43 gm., or less than 5 per cent, was excreted in the urine as glycine nitrogen, and part of this undoubtedly came from the preformed glycine in the proteins of the diet. It was evident that these rats did not synthesize glycine from nitrogenous intermediates to the same extent as the rabbit (13, 15), pig (14), and man (16). If glycine is synthesized from products of protein metabolism, an increase in the protein intake should increase the level of nitrogenous intermediates in the blood and tissues and should, therefore, increase the tolerance of the rat for benzoate. Experiments to test this hypothesis have been unsatisfactory, due apparently to a failure to include sufficient vitamin B in the high protein diets used. Hartwell (17) and Reader and Drummond (18) noted that the vitamin B requirement of young growing rats was greater on high protein diets than on low protein diets. Whether the detoxication of benzoic acid itself increases the vitamin requirement remains to be determined.

SUMMARY.

1. A study was made of the excretion of free benzoic acid, hippuric acid, and total benzoic acid in the urine of rats following the ingestion of sodium benzoate.
2. Hippuric acid constituted 66 to 95 per cent of the total benzoic acid in these urines and was, therefore, the principal product in the detoxication of the ingested benzoate.
3. Survival and growth of young rats on diets containing benzoate only occurred when these diets furnished a supply of glycine which was adequate for the detoxication of the benzoate and for the formation of new tissue proteins.
4. A method for the investigation of the origin of glycine has been described.

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ON THE QUESTION OF THE SPECIFICITY OF THE INTRACELLULAR DEHYDROGENASES.

II. THE EFFECT OF POISONS UPON THE DEHYDROGENASE SYSTEMS OF FROG AND OF FISH MUSCLE.

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(Received for publication, March 8, 1929.)

Several years ago Professor Torsten Thunberg suggested to one of us that the possible specificity of the dehydrogenases of frog muscle might be tested by means of poisons. If there were several specific enzymes, the order of toxicity of certain poisons might be expected to vary widely with the different donators; while if there were a single enzyme of general powers, the same order of toxicity should hold unless the poison reacted with the donator rather than with the enzyme.

The poisons here used were chosen from Thunberg's micro respirometer experiments on unwashed frog muscle, some of which have been reported (3). As some of these poisons are oxidizing or reducing agents, which might attack the methylene blue used in our experiments, the actual amount of interference was determined as follows: To test reduction, vacuum tubes containing methylene blue, buffer, donator, and 0.175 M poison were placed in a water bath at 37° and their color compared at intervals with that of tubes containing various concentrations of methylene blue from zero to the original concentration of 1:200,000. Arsenite reduced the methylene blue by 20 per cent in 2 hours in the presence of citric and lactic acids, but not in the presence of succinic, glycerophosphoric, or L-malic acids. Tellurite reduced the methylene blue little in the 1st hour, and 10 to 40 per cent in the 2nd hour, depending upon the donator. Arsenite and tellurite are therefore of questionable value for our experiments. Phenol reduces methylene blue by 20 to 30 per cent within the 1st hour, without further reduction in the 2nd, but as the amount

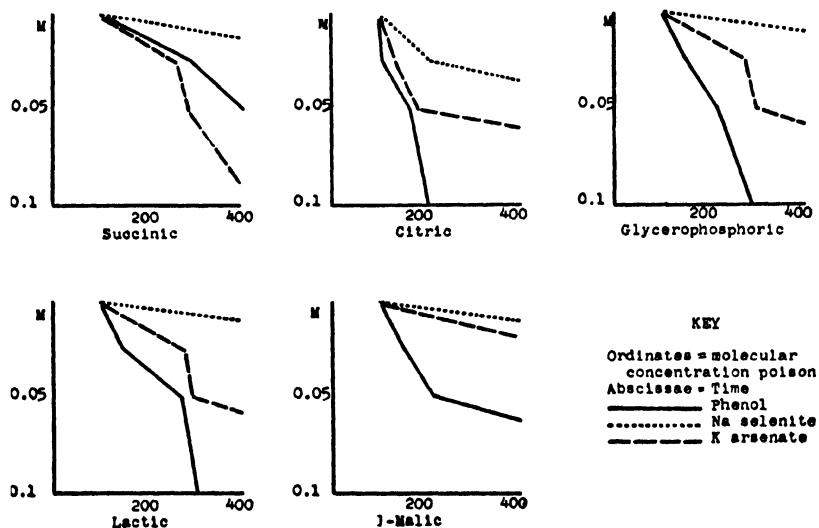


FIG. 1. Relative decolorization time of methylene blue in presence of washed cunner muscle and poisons. Control time = 100.

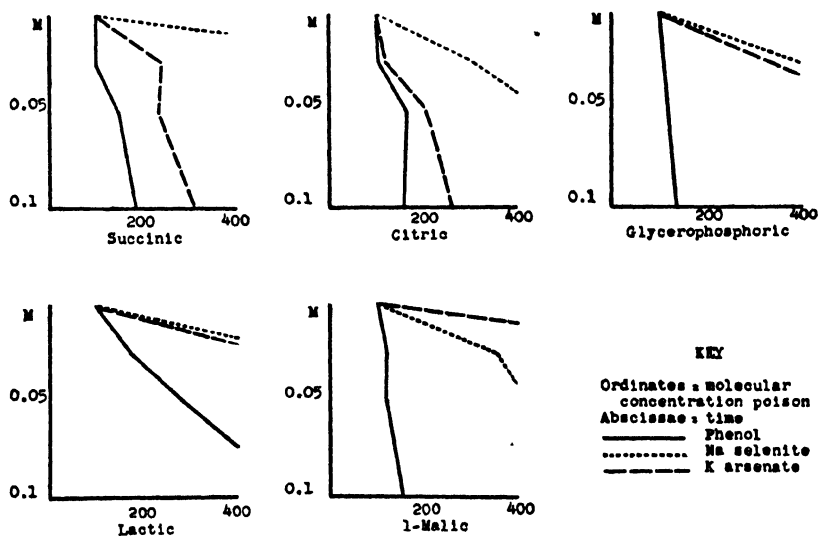


FIG. 2. Relative decolorization time of methylene blue in presence of washed goldfish muscle and poisons. Control time = 100.

of reduction is constant with different donators, it does not greatly affect the value of phenol.

To test oxidation of the methylene blue, unwashed but buffered frog muscle was allowed to reduce methylene blue in U-tubes, through which nitrogen was passed to remove free oxygen. When decolorization was complete, oxygen-free solutions of poisons were added (0.175 M) and the amount of recolorization at 37° noted. Arsenate and chlorate cause no reoxidation of the methylene blue. Iodate and bromate recolorize it by 30 per cent and are

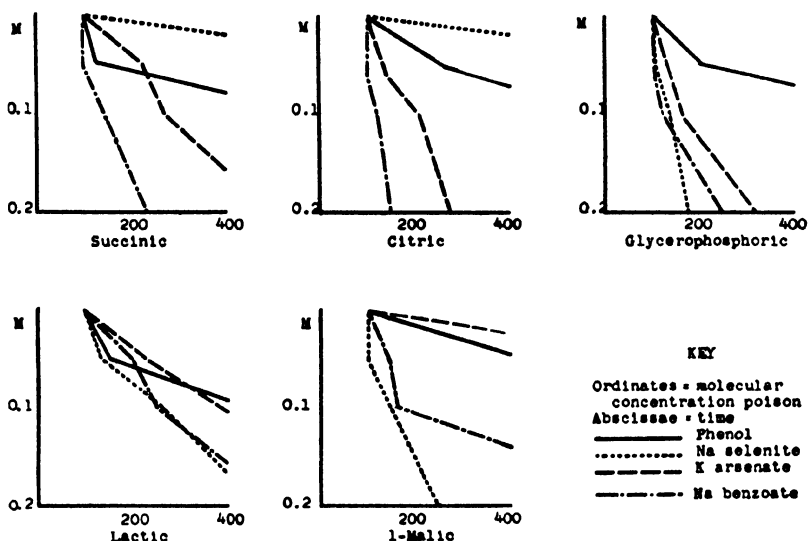


FIG. 3. Relative decolorization time of methylene blue in presence of washed frog muscle and poisons. Control time = 100.

therefore unsuitable. Selenite recolorizes methylene blue very slightly (less than 10 per cent) and so can be used.

This cuts down the original list of poisons to the following: selenite, arsenate, phenol, and benzoate. The technique was like that described in Paper I of this series (2), except that before evacuation poisons were added to the tubes. In order to eliminate poison-donator reactions, the experiments on frog muscle were repeated upon cunner muscle, which is known to contain but one dehydrogenase for the five donators studied.

With washed cunner muscle the order of toxicity with all five

donators was *selenite* > *phenol* > *benzoate*; with citric, glycerophosphoric, and lactic acids, *selenite* > *arsenate* > *phenol*; but with *l*-malic acid the toxicity of arsenate approached that of selenite, and with succinic acid was even less toxic than phenol (Fig. 1).

In order to shorten the decolorization time, which is much longer with fish than with frog muscle, the methylene blue concentration was halved, and part of the experiment repeated with goldfish muscle (Fig. 2). The results were as follows: *selenite* > *arsenate* > *phenol*, except with *l*-malic acid, when it was *arsenate* > *selenite* > *phenol*. Since arsenate is exceptionally toxic with *l*-malic acid in both experiments (cunner and goldfish) it must react with the donator, and so cannot be used to test for the presence of an enzyme specific for this donator.

With washed frog muscle (Fig. 3), the order of toxicity varies considerably between the different donators; *viz.*:

Succinic acid: *selenite* > *phenol* > *arsenate* > *benzoate*.

Glycerophosphoric acid: *phenol* > *arsenate* > *benzoate* > *selenite*.

l-Malic acid: *arsenate* and *phenol* > *benzoate* > *selenite*.

Lactic " *phenol* > *arsenate* > *benzoate* > *selenite*.

Citric " *selenite* > *phenol* > *arsenate* > *benzoate*.

This variation cannot be due to poison-donator reaction (since with fish muscle *selenite* > *phenol* with all five donators), and must therefore indicate the presence of more than one dehydrogenase. Again, arsenate is nearly equal in toxicity to phenol with lactic and *l*-malic acids, but is much less toxic than phenol with succinic, citric, and glycerophosphoric acids. If we eliminate *l*-malic and succinic acids because of arsenate-donator reactions noted with cunner muscle, there remain three sound cases in which variation must be due to poison-enzyme reaction.

If the grouping of the donators in regard to these three poisons (*selenite*, *arsenate*, *phenol*) be compared, it is clear that in the one case (*selenite* and *phenol*) they fall into two groups, glycerophosphoric, lactic, *l*-malic *versus* succinic and citric acids; while in the second case (*arsenate* and *phenol*) the two groups are different; *viz.*, lactic acid *versus* citric and glycerophosphoric acids. This indicates that at least three dehydrogenases are present; *viz.*, citric, glycerophosphoric, and lactic. It is also significant that the succinic acid reaction, which is much more

resistant than the others to a variety of treatments (1), shows itself in the experiments with selenite to be distinctly less resistant than lactic, *L*-malic, or glycerophosphoric acids. A larger number of suitable poisons must be used in order to extend the proof to other dehydrogenases.

SUMMARY.

Poisons, which with fish muscle show the same order of toxicity with the donators here studied, vary significantly in toxicity with frog muscle. As these variations are not due to poison-donator reactions, they must be due to poison-enzyme reactions, and therefore indicate the presence of at least three specific dehydrogenases; *viz.*, citric, glycerophosphoric, and lactic.

We are greatly indebted to Professor Torald Sollmann, of the Department of Pharmacology, School of Medicine, for his hospitality in giving us laboratory facilities, and for his constant interest and advice. We are grateful also to Miss Joyce McGavran for assistance in some of the experiments.

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ON THE QUESTION OF THE SPECIFICITY OF THE INTRACELLULAR DEHYDROGENASES.

III. THE DEHYDROGENASES OF FROG MUSCLE.

By MARY E. COLLETT, MIRIAM F. CLARKE, AND
JOYCE McGAVRAN.

(From Western Reserve University, Cleveland.)

(Received for publication, March 8, 1929.)

The surest test of specificity is obtained by comparing the decolorization times when optimal concentrations of two donators are used singly or in pairs. The addition of a second donator will not increase the rate of reduction if only one enzyme is present, but must increase it if each of the donators is attacked by a different enzyme. Thus far several studies of the sort have been reported, but little has been done on frog muscle.

Ahlgren (1) found an additive effect with succinic and lactic acids in frog muscle, and with succinic and glycerophosphoric acids in guinea pig muscle. Quastel and Wooldridge (6) tried many donators with resting *Bacillus coli*; in most cases there was no addition, but in a few definite addition was observed. In their experiments upon toluene-treated *Bacillus coli* (7) they found four specific adsorption centers (for succinic, lactic, formic acids, and dextrose) which may represent specific enzymes. Several specific dehydrogenase extracts have been reported recently; *Bacillus coli*, lactase (8); mammalian liver, citrase (3) and fumarase (4); yeast, lactase (3); frog muscle, fumarase and succinase (2). There is therefore ample evidence from a variety of sources for the existence of specific dehydrogenases, but, since the evidence is scattered, it seems worth while to continue the work begun by Ahlgren on frog muscle in order to round out the work already done on that tissue.

The only variations from the technique already described are that the muscle was washed in normal saline as well as in distilled water, and that the concentration of the methylene blue was

trebled and the temperature lowered to 32° in order to slow the reaction.

Table I shows the results observed with succinic, citric, glycerophosphoric, lactic, and *l*-malic acids as donators. It is evident that the decolorization time for every pair is shorter than for either member of the pair taken alone, which means that in frog muscle there are specific dehydrogenases, one for each of the donators tested. This agrees with Ahlgren's single experiment upon frog muscle. It is noteworthy that frog muscle, in possessing several dehydrogenases, differs from fish muscle which contains only one (5).

TABLE I.

Decolorization Time of Methylene Blue by Washed Frog Muscle in Presence of Optimal Concentrations of Donators, Used Singly or in Pairs.

Methylene blue 3:100,000. Temperature 32°.

Date.	Donators.		A	A+B	B
	A	B			
1928					
Nov. 26	Succinic	(0.01 M) and citric (0.01 M).	17½	12	20
Dec. 10	"	(0.01 " " l-malic (0.02 ").	22½	14	20
" 12	"	(0.01 " " lactic (0.02 ").	23	19	23
" 1	"	(0.01 " " glycerophosphoric (0.03 M).	15	6½	10½
Nov. 26	Citric	(0.01 " " l-malic (0.02 M).	24	15	20½
Dec. 12	"	(0.01 " " lactic (0.02 ").	23	18	23
" 12	"	(0.01 " " glycerophosphoric (0.03 M).	18	9	16
" 1	Lactic	(0.02 " " " (0.03 ").	24	16½	22
" 13	"	(0.02 " " l-malic (0.02 M).	27	12	20
" 1	l-Malic	(0.02 " " glycerophosphoric (0.03 M).	17½	8	15½

SUMMARY.

Since the reaction time is hastened by the addition of a second donator in optimal concentration, we conclude that frog muscle contains specific dehydrogenases, one for each of the five donators tested.

We are greatly indebted to Professor Torald Sollmann, of the Department of Pharmacology, School of Medicine, for his hospitality in giving us laboratory facilities.

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THE CONDENSATION OF AROMATIC ALDEHYDES WITH GLYCINE AND ACETYLGLYCINE.

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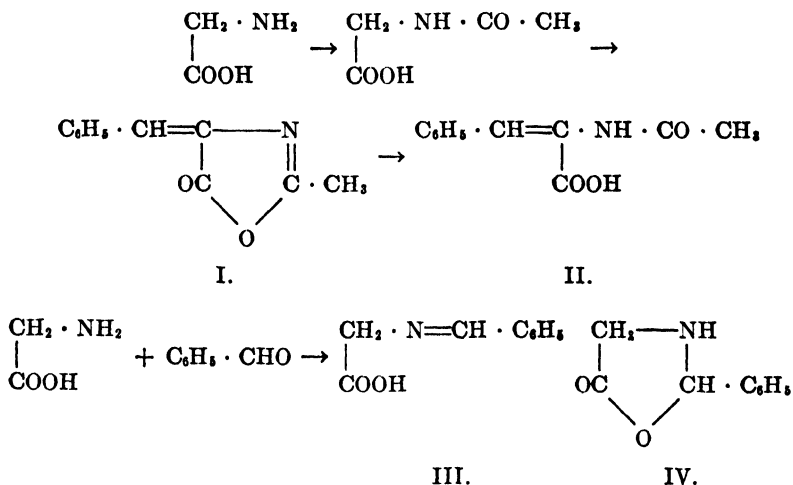
(Received for publication, March 29, 1929.)

The condensation of aromatic aldehydes with acyl derivatives of glycine has, in its various modifications, furnished the most useful method available for the synthesis of compounds which may in turn be converted into aromatic amino and ketonic acids such as tyrosine, tryptophane, and thyroxine. The reaction was first studied by Plöchl (1) who condensed hippuric acid with benzaldehyde and salicylic aldehyde. The correct interpretation of this reaction and the demonstration of its ability for amino acid synthesis was furnished later by Erlenmeyer (2). Instead of hippuric acid, later investigators (3) have used other derivatives of glycine such as hydantoin and glycine anhydride. In connection with an investigation of aldehyde derivatives of amino acids, peptides, and proteins it became of interest to examine the behavior of glycine itself when condensed with benzaldehyde and acetic anhydride. The reaction was first studied by Plöchl (1) who stated that: "Es konnten jedoch daraus auf keine Weise zur Untersuchung einladende Körper sondern nur Schmierer erhalten werden." In fact it was this failure to effect the condensation of glycine that led Plöchl to use hippuric acid in its place. Later on Erlenmeyer and Früstück (4) succeeded in isolating the azlactone of α -acetaminocinnamic acid and subsequently Bergmann and Stern (5), by varying the conditions, obtained 44 to 50 per cent yields and showed how valuable this and the related derivative from *p*-hydroxybenzaldehyde were for the synthesis of amino acids and peptides.

A reinvestigation of the condensation just referred to has shown that the reaction is complicated by a competing reaction taking place between glycine and benzaldehyde with formation of a non-

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acetylated derivative possibly of a type resembling a Schiff base. The two reactions may be represented as follows:

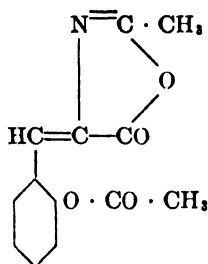


This substance (III) which may be called benzylidene glycine was isolated and analyzed. By analogy and in view of its relative stability it would appear not unlikely that the substance is polymerized though accurate molecular weight determinations are lacking. It is also possible that the alternative structure (IV) containing the same ring as the azlactones may demand consideration. Analogous compounds have been obtained from other amino acids and will be described later.

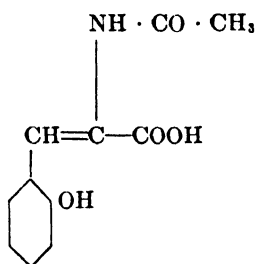
In view of the fact that this second product of the condensation of glycine and benzaldehyde with acetic anhydride is not an acetylated compound, it appeared likely that its formation could be largely suppressed by acetylating the glycine before subjecting it to reaction with the benzaldehyde. This proved to be the case and correspondingly larger yields of the azlactone of α -acetaminocinnamic acid were obtained. The acetylation of glycine is not an entirely simple matter. Boiling with glacial acetic acid is ineffective, while warming on the water bath with excess of acetic anhydride yields colored products for the removal of which Radenhausen (6) advocated the use of chlorine. It was found that the acetylation could be far more conveniently effected by warming glycine suspended in 3 parts of glacial acetic acid with

the theoretical amount of acetic anhydride until solution is just effected. This only takes a minute or so with moderate quantities of materials and on cooling a virtually theoretical yield of acetylglycine (aceturic acid) crystallizes out. This method offers by far the easiest method of preparation of the substance. The acetylated glycine was then condensed with a variety of typical aldehydes as described in the experimental part of this paper.

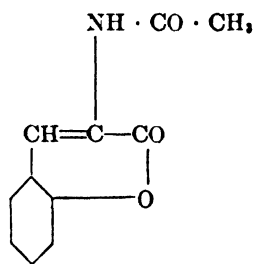
The only product of the reaction calling for any comment is that derived from salicylic aldehyde. While the product first formed is the normal acetylated azlactone (V), on treatment with



V.



VI.



VII.

alkali and subsequent acidification the expected *o*-hydroxy- α -acetaminocinnamic acid (VI) has only a transient existence and passed over to a neutral substance giving no phenolic reactions, which is doubtless α -acetaminocoumarin (VII).

A word may be added as to the use of the reaction just described for preparative purposes. As regards the synthesis of aromatic amino acids and peptides, their utility has been admirably shown by Bergmann and Stern. For the preparation of α -ketonic acids the reaction should prove of real value. The ordinary Erlenmeyer method of preparation by the action of strong alkali or azlactones derived from hippuric acid is always complicated by the simultaneous formation of benzoic acid, the complete separation of which from the α -ketonic acid is often a matter of doubt and difficulty. This objection is entirely avoided by the use of the azlactones derived from acetylglycine. These compounds on treatment with alkali give α -ketonic acids and acetic acid very smoothly in good yield and their separation offers no difficulty.

EXPERIMENTAL.

Direct Condensation of Glycine with Benzaldehyde.—Glycine (2 gm.), anhydrous sodium acetate (2 gm.), benzaldehyde (5 gm.), and acetic anhydride (10 cc.) were heated in a water bath for 2 hours. Water was then added and the excess of benzaldehyde blown off with steam. Sulfuric acid was then added until acid to Congo red and the clear aqueous solution poured off from the yellow-brown solid material. This solution on standing deposits a considerable amount of α -acetaminocinnamic acid. The solid matter was then dissolved in half normal sodium hydroxide and filtered from a trace of residue. The filtrate is acidified and the precipitate filtered off, washed, and dried. It is made up of a large amount of α -acetaminocinnamic acid with a smaller amount of benzylidene glycine. The latter is separated by dissolving the mixture in a minimum amount of absolute alcohol and precipitating with dry ether. A brownish precipitate separates out which may be purified by repeating the precipitation. The yield of this product is about 10 per cent but undoubtedly much is lost. A yield of about 50 per cent of α -acetaminocinnamic acid is obtained from the ether mother liquors. Its properties are identical with those previously described.

Benzylidene glycine forms a buff-colored powder which gives a brown solution in alcohol. Its alcoholic solution reacts acid to phenolphthalein but only requires about half the theoretical amount of alkali to neutralize it. It is insoluble in water, ether, chloroform, and petroleum, but when freshly prepared is freely soluble in alcohols and in aqueous sodium hydroxide. It has not been possible to crystallize it and the analytical data constitute the only evidence of its purity. Several preparations of identical properties were made. On heating, the substance darkens at about 180° and melts somewhat indefinitely at about 207° . As stated in the introduction it is not unlikely that the substance is polymerized and its structure cannot be regarded as settled. For analysis the product was dried in a vacuum at 80° over phosphorus pentoxide. It does not appear to be hygroscopic.

Analysis. $C_9H_9O_2N$.

Calculated. C 66.4, H 5.52, N 8.59.

Found. " 66.4, 66.6, " 5.74, 5.72, " 8.2, 8.40.

Acetylglycine (Aceturic Acid).—Finely powdered glycine is suspended in 4 cc. of glacial acetic acid and acetic anhydride (2.0 cc.) added. The mixture contained in a small flask is gently rotated over a small flame until the glycine is just dissolved. This requires only about a minute and prolonged heating must be avoided. On cooling, crystals of aceturic acid separate out at once. The product may be sucked off, washed with a little water or ether, and dried. The yield amounts to 2.15 to 2.20 gm., over 90 per cent of the theoretical amount, and requires no further purification. It melts at 206°. On titration with phenolphthalein 0.1 gm. required 8.6 cc. of decinormal sodium hydroxide, indicating a molecular weight of 116 as against a calculated value of 117. It also gave satisfactory results on elementary analysis.

Az lactone of α -Acetaminocinnamic Acid.—Glycine (1.5 gm.) was acetylated as above described by use of 3 cc. of glacial acetic acid and 2.0 cc. of acetic anhydride. Without removing the acetic acid mother liquor from the acetylglycine, 1.6 gm. (1 mol) of anhydrous sodium acetate were added together with benzaldehyde, 2.2 gm. (1 mol), and acetic anhydride, 7 cc. The mixture was heated for 2 hours in a water bath. No separation of crystals takes place during the heating but the color changes to a dark yellowish green. On adding water by degrees to the cooled mixture the az lactone is precipitated as a yellow mass. It is filtered off, spread on porous plates, and dried. The yield of crude product amounts to 70 per cent of the theoretical quantity. It is purified by recrystallization from benzene which leaves behind a trace of insoluble impurity. The substance formed stout yellow needles melting sharply at 154–155°. Bergmann and Stern record 151–152°, while Erlenmeyer and Früstück gave 146–147°. The substance gave satisfactory results on analysis. The preparation of the above compound is unfavorably influenced by too prolonged heating or by using a higher temperature. Acetic acid without acetic anhydride is not effective.

α -Acetaminocinnamic Acid.—On dissolving the preceding az lactone in hot 0.5 N sodium hydroxide (3 mols) and acidifying the filtered solution, this compound readily separates. It is recrystallized from boiling water, a little charcoal being used to remove pigment. It is readily soluble in hot water, sparingly in cold, and forms colorless cubes and plates melting at 190–192° as described

by Bergmann and Stern. It reduces permanganate freely and gives phenylpyruvic acid on boiling with 20 per cent sodium hydroxide.

Azlactone of o-Acetoxy- α -Acetaminocinnamic Acid.—This compound was readily obtained from salicylic aldehyde. The preparation was carried out exactly as for the preceding azlactone except that 10 cc. of acetic anhydride were used and 2.4 gm. of salicylic aldehyde. The yield of crude product amounted to 2.3 gm. It was recrystallized from toluene, too long boiling with the solvent being avoided. It crystallizes in long bright yellow needles, moderately soluble in hot alcohol, sparingly soluble in cold alcohol. It melts at 203–205°.

*Analysis.** $C_{13}H_{11}O_4N$.

Calculated. C 63.7, H 4.49, N 5.71.

Found. " 63.5, " 4.72, " 5.65.

* The analyses for carbon and hydrogen recorded in this paper were all made in the usual way with 120 to 150 mg. of substance. The nitrogen determinations were made by Kjeldahl's macro method.

α -Acetaminocoumarin.—On dissolving the preceding substance in three molecular proportions of half normal sodium hydroxide and then precipitating the bright red alkaline solution with mineral acid, the initial precipitate which presumably is *o*-hydroxy- α -acetaminocinnamic acid rapidly changes to a heavy brown neutral compound. It is very sparingly soluble in water but may be recrystallized conveniently from methyl alcohol with a little charcoal. It forms felted masses of long colorless needles melting at 203–204°.

Its aqueous solution reacts neutral to litmus, and it gives no coloration with ferric chloride or Millon's reagent. Its properties and analysis clearly indicate that the substance is α -acetaminocoumarin (VII).

Analysis. $C_{11}H_9O_3N$.

Calculated. C 65.0, H 4.75, N 6.82.

Found. " 64.9, " 4.43, " 6.90.

Azlactone of p-Acetoxy- α -Acetaminocinnamic Acid.—The condensation was carried out as in the case of salicylic aldehyde. The yield was 72 per cent of the theoretical amount. The azlactone

is readily crystallized from methyl alcohol in which it is freely soluble in the boiling solvent but sparingly soluble at room temperatures. It forms glistening golden plates melting at 138–139°+. Bergmann and Stern record 131–132°.

p-Hydroxy- α -Acetaminocinnamic Acid.—On dissolving the preceding substance in half normal sodium hydroxide and then acidifying, the free acid separates rather slowly in needles that are apt to retain a little pigment. On recrystallizing from water with charcoal, colorless clear lance-shaped needles are readily obtained. It is sparingly soluble in cold water, freely soluble in hot water or alcohol. The substance dried at room temperature contains water of crystallization and when heated softens around 140° before melting at 207°. On drying at 110°, the clear crystals become opaque and then melt sharply at 203–205° as described by Bergmann and Stern.

Azlactone of α -Acetaminopiperonylacrylic Acid.—Piperonal (3 gm.), used in place of benzaldehyde, gave after 3 hours heating on the water bath 2.60 gm. of condensation product. It was well washed with water and then treated with a little ether to remove a trace of oily impurity. It was recrystallized from toluene and forms glistening prisms with a greenish gold color. It is sparingly soluble in methyl alcohol even when hot, freely soluble in acetic acid and ethyl acetate, sparingly soluble in ether. It melts at 183–184°.

Analysis. $C_{12}H_9O_4N$.

Calculated. C 62.4, H 3.89, N 6.06.

Found. " 62.4, " 4.20, " 6.10.

α -Acetaminopiperonylacrylic Acid.—The free acid was obtained in the usual way from the preceding azlactone. It was recrystallized from water, in which it is sparingly soluble even at the boiling temperature. It separates in the form of nodular masses of needles concentrically arranged and melts at 220–221°.

Analysis. $C_{13}H_{11}O_5N$.

Calculated. C 57.8, H 4.43.

Found. " 57.3, " 4.59.

*Azlactone of α -Acetamino-*p*-Nitrocinnamic Acid.*—*p*-Nitrobenzaldehyde (3.22 gm.), when treated as previously described, gave 96 per cent of the theoretical amount of condensation product.

In the early stage of the reaction, solution is complete but after longer heating on the water bath a solid cake of crystals separates out. This crystallization was not observed in the case of any of the related compounds here described. After an hour of heating, water was added and the compound filtered off. It is sparingly soluble in alcohol or benzene but can be readily crystallized from acetic acid. The crystals have a fine clear orange-yellow color and have the shape of long whetstones. The melting point is 185–186°.

Analysis. $C_{11}H_8O_4N_2$.

Calculated. C 56.8, H 3.45, N 12.0.

Found. " 57.1, " 3.58, " 11.8.

α-Acetamino-p-Nitrocinnamic Acid.—The preceding azlactone dissolves in dilute sodium hydroxide with a clear red color and on acidifying the acid is obtained as an almost white granular precipitate. It is very sparingly soluble in water but readily soluble in alcohol. Dilute methyl alcohol is a convenient solvent for recrystallization. It forms sickle-shaped crystals melting at 234–235°.

Analysis. $C_{11}H_{10}O_5N_2$.

Calculated. C 52.8, H 4.00, N 11.2.

Found. " 53.2, " 4.32, " 11.4.

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PLANT HEMAGGLUTININS WITH SPECIAL REFERENCE TO A PREPARATION FROM THE NAVY BEAN.*

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Since the discovery of ricin by Kobert in 1887, considerable interest has been shown in the hemagglutinins obtainable from plants. The study of them has been somewhat overshadowed by the more recently discovered serum agglutinins, which they so remarkably resemble in action. They have, however, played an important part in immunological research throughout its history.

Ricin, the extremely toxic hemagglutinin from the castor bean (*Ricinus communis*) was used by Ehrlich (1897) in his first *in vitro* studies of the reactions between antigen and antibody.¹ The living organism was no longer to be regarded as an indispensable participant in the agglutinin-antiagglutinin reaction.

Much remains to be done before the distribution of the hemagglutinins in the plant world can be estimated. From the observations made by Landsteiner and Raubitschek (1908), Mendel (1909), Eisler and Porthelm (1909, 1926), Wienhaus (1909), and others, the substances appear to lack wide distribution.² The early studies were confined to the toxic agglutinins: ricin, robin, crotin, and abrin. Later, non-toxic extracts were prepared in

* The data reported in this paper are taken from the dissertation presented by Verz R. Goddard in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University, 1927.

¹ He showed that ricin neutralized by antiricin would not agglutinate rabbit corpuscles in a test-tube. From quantitative work, he concluded that this neutralization followed the law of multiple proportions.

² A detailed summary of the principal plant sources which have been investigated with reference to the occurrence of hemagglutinins and of the animal species furnishing the blood corpuscles tested is included in the dissertation of Verz R. Goddard, Yale University, 1927.

various laboratories. The seeds of leguminous plants are especially rich in potent products.

As blood cell clumping substances, phytohemagglutinins apparently have no relationship to the activity of the plant producing them. This is analogous to the well known fact that plants produce enzymes having no obvious bearing upon the activities of the structures that yield them. The presence of pancreatic secretin in spinach and of urcase in beans are obvious examples.

A possible physiological relationship between the agglutinin and the plant producing it has been indicated by Eisler and Portheim (1911, 1912, 1926) and by Schneider (1912). The hemagglutinin appears to be associated with the storage material of plants. Upon germination the seed loses its agglutinin which may pass over to the embryo (Russ and Oesterlin, 1921). It is interesting to note that certain plants which have reserve material in their stems, possess an agglutinating milk-sap. This is true of the *Euphorbia* studied by Eisler and Portheim. More recently, Marcusson-Begun (1926) has reported an agglutinin in the potato and has suggested the possibility of the occurrence of agglutinating substances in other root nodules.

The classic agglutinin-containing seed, that of the castor bean (*Ricinus communis*) was studied chemically by Osborne, Mendel, and Harris (1905), who made a separation of its proteins. Both the toxic and the hemagglutinative qualities were found to be associated with the albumin, ricin, a name earlier indicated by Kobert for the toxic principle. Their method for the purification of ricin may be used for its preparation on a large scale (Karrer, Smirnoff, Ehrensperger, van Slooten, and Keller, 1924).

For many purposes a non-toxic agglutinin is of value. Such a substance might well prove of considerable practical importance as an aid in the preparation of antisera. In fact, a crude extract of the pea-bean (*Phaseolus communis*) is being used in the preparation of certain of the anti hog cholera sera on a commercial scale (Dorset and Henley, 1916). It has been shown that where a clear, corpuscle-free serum is desired, the yield obtainable from a given volume of blood can be notably increased by the use of this agglutinin which causes the corpuscles to pack and makes possible a nearly perfect separation of cells and serum.

The present study involves the preparation of a non-toxic

hemagglutinin in a highly purified form and an investigation of the mechanism of its action in the hope of making the material more useful in research as well as for practical, serological work. The applicability of such a substance is, of course, not limited to immunological research. It can serve in various capacities in the study of the physical characteristics of erythrocytes and possibly of tissue cells in suspension. Wienhaus (1909) has shown that a bean extract would cause agglutination of pus, liver, and kidney cells.

Preparation of Hemagglutinin and Other Experimental Procedures.

For the preparation of the water-soluble protein fraction of the bean (*Phaseolus communis*) the method employed by Osborne, Mendel, and Harris in the preparation of ricin was closely followed. The influence of alterations in the integrity of the protein upon the hemagglutinating properties was also investigated.

After extraction of the defatted bean meal with sodium chloride solution (usually 3 per cent), removal of the undesired globulins by dialysis of the extract, repeated salting out of the desired protein with ammonium sulfate, and dialysis of the precipitate to remove inorganic contaminants, a clear, straw-colored filtrate was obtained which was evaporated in a current of air to dryness at 35–40° on glass plates. The protein was then scraped from the plates, further desiccated over sulfuric acid, and stored in the form of a nearly white powder in glass bottles. Upon analysis the samples, with corrections for moisture and ash, varied in nitrogen content from 14.5 to 15.5 per cent. The material responded positively to all the common protein color and precipitation tests. This substance had a high hemagglutinating potency. A sample which has been stored for over 2 years still shows (August, 1928) no appreciable loss in activity. Injections of as much as 8 mg. per kilo into rabbits and 600 per kilo into mice indicated that the material is non-toxic, in contrast to the comparable ricin preparation from the castor bean for which a lethal dose as low as 0.002 mg. has been observed.

Macroscopic Agglutination Method.—Many macroscopic methods for detecting agglutination of bacteria have been published and a plea has been made for standardization of reports of agglutination tests (Hadley, 1916). Most of these procedures are based

upon the Kolmer technique (Kolmer, 1923). Gardner's gelatin standard method (Gardner, 1919) and Gates' capillary tube method (Gates, 1921) represent special modifications. The difficulty in detecting agglutination appears to arise from the fact that it is a two phase reaction, the binding of the agglutinating agent occurring previous to flocculation. Gates (1922) has suggested a method for eliminating this time element in the agglutination of bacteria. He hastened the process by centrifuging the organisms in contact with the agglutinin. A similar technique was recommended by Mudd (1927). The centrifuge method was found, in the experience of the writers, to give reasonably good results with erythrocytes also. After centrifugation agglutinated cells would remain in the bottom of an upturned tube or if agglutination were slight, would stream very slowly in comparison to the controls.

In an attempt to devise a procedure, however, which would give completely consistent results in the quantitative study of hemagglutination, factors known to be significant in clumping and sedimentation were taken into consideration. Temperature, diameter of tube and length of liquid column, angle or slope of tube were regulated to give the best results obtainable. A chance observation led to the development of a method which proved invaluable in determination of agglutination titer.

It was found in agreement with Berczeller and Wastl (1923) that slanting the tube increased the rate of sedimentation. But it was noted, also, that after erythrocytes had been left in contact with agglutinin in chemically clean glass tubes, sloped at an angle of 40° and at a temperature of $40-44^{\circ}$, for 12 hours, the cells became firmly attached to the glass so that upon returning the tube to the vertical position they remained unmoved. This peculiar adherence of cells never occurred in the absence of hemagglutinin nor when the latter was too dilute to bring about clumping detectable upon microscopic examination.

The ease by which hemagglutination is detected by this method is indicated in Fig. 1. Note the appearance of tubes in which the same volumes of rabbit erythrocytes, washed and suspended in physiological salt solution, were treated with increasing quantities of hemagglutinin. In the tube furthest left and where no agglutination occurred the corpuscles settled; the other tubes show

clearly the adherence of the red cells characteristic of agglutination upon the glass walls.

After various modifications had been investigated, the following procedure was finally selected as most effective. Rabbit blood freshly removed from the ear vein and defibrinated was centrifuged until the serum could be removed. The corpuscles washed twice with 0.89 per cent sodium chloride solution were suspended therein. 2.5 cc. of packed corpuscles were suspended in a total volume of 100 cc. of the isotonic sodium chloride solu-

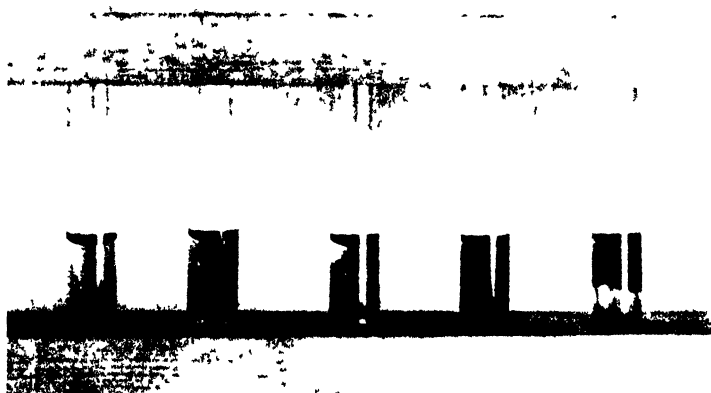


FIG. 1 Hemagglutination test; one negative (left), four positive agglutinations (right). Tubes contained (left to right): 0.0005, 0.0006, 0.0007, 0.0008, and 0.0009 mg. of hemagglutinin with 0.5 cc. of 2.5 per cent suspension of rabbit erythrocytes in sodium chloride solution. (Each tube was made up to a total volume of 1 cc.)

tion. Aliquots of this suspension were used for the individual tests. As a rule 0.5 cc. of the corpuscle suspension was added to 0.5 cc. of 0.89 per cent sodium chloride solution containing varying amounts of the agglutinin preparation to be tested. The reaction was carried out in chemically clean glass tubes, 75×8 mm., placed in a rack which would keep them at an angle of 40° and incubated at $40-44^\circ$, for 12 hours. Table I indicates the uniformity of results secured by this method of "agglutination titer" when tests were repeated on different days, with fresh suspensions of eryth-

TABLE I
Measure of Agglutinating Activity.

Date of experiment	Minimum effective quantity of hemagglutinin.
<i>1926</i>	<i>mg.</i>
Mar. 14	0 0004
" 15	0 0006
" 18	0 0006
" 24	0 0006
" 31	0 0006
Apr. 9	0 0005
" 9	0 0006
" 12	0 0005

The hemagglutinin was added to 0.5 cc. portions of a 2.5 per cent suspension of washed, rabbit erythrocytes in a total volume of 1 cc.

TABLE II.
Agglutinability of Erythrocytes of Different Species.

Date of experiment	Kind of erythrocyte	Minimum effective concentration of agglutinin
<i>1927</i>		<i>mg</i>
Mar. 15	Rabbit.	0 0006
" 28	Man.	0 0007
" 30	"	0 0008
" 31	"	0 0007
Apr. 13	"	0 0007
Mar. 20	Duck	0 0008
Apr. 15	Dog.	0 0008
" 15	"	0 0008
" 29	"	0 0009
" 29	Mouse.	0 002
May 4	"	0 002
" 9	Rat.	0 003
Mar. 20	Chicken.	0 006
Apr. 17	"	0 006
" 17	"	0.006

To 0.5 cc. portions of 2.5 per cent suspensions of the washed erythrocytes in isotonic sodium chloride were added 0.5 cc. portions of isotonic sodium chloride containing the quantity of hemagglutinin to be tested.

rocytes. In Table II the results of comparing the agglutination titers of different species of animals are shown.

From Tables I and II it can be seen that the dilution at which the agglutinin is still active is, for rabbit erythrocytes, 1 to 6,000,000 or by analogy with ordinary immunological terminology this figure could be called its titer, since clumping is effected in a total volume of 1 cc. of solution containing only 0.0006 mg. of agglutinin by dry weight. It is active in lower concentration than the purified bean agglutinin "phasin" as reported by Wienhaus in 1909. His product was, however, only partially soluble, having suffered considerable denaturation during the process of preparation. It seems probable that the soluble fraction of his material was highly active. In contrast to Wienhaus' phasin, the bean agglutinin reported here gave remarkably similar results with the washed corpuscles of the various species tested—especially with those of man, rabbit, dog, and duck. The erythrocytes of the hen, more difficult to clump than the other species tried, required only 10 times as much agglutinin as was necessary for rabbit.

Influence of Medium upon Hemagglutination.

Inhibiting Factors.—Not only artificial media, but the natural medium of the erythrocytes, *i.e.* blood serum, shows a strong influence upon agglutination of the corpuscles. This was noted by Landsteiner and Raubitschek in their early work. Raubitschek (Kraus and Levaditi, 1911) was of the opinion that the inhibiting influence of serum is due to the presence of its proteins. He noted the selective affinity of erythrocytes for certain substances, such as peptones and albumoses. Agglutinated cells shaken and incubated with peptone solution became unagglutinated. Our own observation supports Raubitschek's conclusion regarding the retarding influence of protein material upon the agglutination reaction. Table III indicates the effect of egg white upon the action of the agglutinin preparation. In these experiments the standard method already described was not employed. Whole defibrinated blood in 0.1 cc. portions was treated with solutions of bean hemagglutinin dissolved in 0.89 per cent sodium chloride in a total volume of 1 cc. Column 1 shows the effect of the hemagglutinin alone; the results in Column 2 were secured when egg white was

introduced just after the addition of the hemagglutinin, whereas in experiments in Column 3 the egg white was added just prior to the addition of hemagglutinin. When the corpuscles were first treated with egg white they failed to agglutinate in the presence

TABLE III.

Influence of Egg White upon Agglutination of Whole, Defibrinated Blood.

Hemagglutinin added.	Effect upon hemagglutination.		
	Blood + hemagglutinin. (1)	Blood + hemagglutinin + 1 cc. egg white. (2)	Blood + 1 cc. egg white + hemagglutinin. (3)
<i>mg.</i>			
0.05	—	—	—
0.06	+	—	—
0.07	+	+	—
0.08	+	+	—
0.09	+	+	—

TABLE IV.

Inhibitory Influence of Serum upon Hemagglutination.

Suspension.	Kind of erythrocytes.	Kind of serum.	Volume of serum.	Minimum effective quantity of agglutinin.
<i>vol. per cent</i>			<i>cc.</i>	<i>mg.</i>
2.5	Rabbit.	None.		0.0006
2.5	"	Rabbit.	0.012	0.002
2.5	"	Turtle.	0.012	0.006
5.0	"	Rabbit.	0.025	0.003
5.0	Duck.	None.		0.0008
5.0	"	Duck.	0.025	0.002
5.0	"	Hen.	0.025	0.006

0.5 cc. portions of a suspension of washed corpuscles were used in a total volume of 1 cc. with and without additions of equal volumes of serum, the suspension being diluted with isotonic saline solution to a total volume of 1 cc. Before dilution to final volume, serum was added in a proportion equivalent to the actual volume of the corpuscles.

of far greater quantities of hemagglutinin than were readily effective without the egg white. When the egg white was added after the corpuscles and the agglutinin had had even a brief opportunity to act the influence was less marked.

The effect of adding traces of blood serum to washed corpuscles can be seen from Table IV, a summary of a series of experiments in which washed corpuscles suspended in saline were compared with those to which serum of the same or foreign species had been added. Serum of the turtle and the hen were particularly inhibitory. For rabbit serum the ratio between the requisite

TABLE V.
Influence of Sodium Chloride upon Hemagglutination

Date of experiment	Erythrocyte suspension	Agglutinin added	Minimum effective weight of NaCl
1926	vol per cent	mg	mg
Apr. 10	5 0	0 002	>0 7
" 10	5 0	0 005	0 4
" 10	10 0	0 005	0 4
May 15	2 5	0 02	0 3
" 17	2 5	0 03	0 2
" 17	2 5	0 04	0 2
" 16	2 5	0 05	0 2
Apr 9	2 5	0 05	0 5
May 17	2 5	0 08	0 2
" 17	2 5	0 08	0 2*
" 16	2 5	0 1	0 2
" 17	2 5	0 1	0 2
" 16	2 5	0 15	0 2

Summary of a series of experiments in each of which the amount of hemagglutinin was kept constant and sodium chloride was varied in different test samples until agglutination was obtained. Samples consisted of 0.5 cc. portions of a suspension of washed corpuscles in isotonic sucrose, diluted to a total volume of 1 cc. with an isotonic mixture containing known weight of hemagglutinin and the quantity of sodium chloride to be tested.

*After the corpuscles of this series had been allowed to react with agglutinin for $\frac{1}{2}$ hour, they were washed with sucrose solution to remove excess agglutinin, resuspended in sucrose solution, and titrated, with the result indicated.

amounts of agglutinin for clumping equivalent volumes of erythrocytes in whole, diluted blood and in sodium chloride solution was approximately 5:3.

Favoring Influence.—Electrolytes are of course factors in the medium essential for hemagglutination. Rona and György (1920) concluded from their work on the influence of electrolytes upon

ricin agglutination that a definite cation series could be established by arranging the salts in the order of their values. With the hemagglutinin reported here, it was found that when the concentration of electrolyte was greatly lowered, excessively large amounts of agglutinin were required. Thus, corpuscles washed three times in 0.25 M sucrose solution required 10 times as much hemagglutinin as those washed and suspended in isotonic sodium chloride solution. After washing the cells twelve times in isotonic sucrose solution, it was no longer possible to cause agglutination with any concentration of protein used.

In order to study the relationship between concentration of hemagglutinin and quantity of electrolyte, a series of media was prepared containing mixtures of varying concentrations of sodium chloride and sucrose selected to keep them isotonic with rabbit erythrocytes. These were used in a series of "titration experiments" summarized in Table V. To 0.5 cc. portions of a 2.5 per cent suspension of rabbit erythrocytes in isotonic sucrose were added 0.5 cc. portions of sucrose solution containing the amount of agglutinin indicated with varying quantities of sodium chloride. Table V records the minimum weight of sodium chloride in the presence of which agglutination is brought about in each case. The "titration" was not carried far enough to determine the concentration of sodium chloride necessary to permit agglutination of the corpuscles with 0.002 mg. of protein. It was greater than 0.72 mg. of sodium chloride. As the weight of hemagglutinin increased, the requirement for sodium chloride decreased until the value of hemagglutinin reached a certain level, *i.e.* 0.02 mg. Beyond this value, the quantity of sodium chloride required was shown to be constant—0.2 mg. Isotonic salt solution would contain in an equal volume about 10 times this concentration of sodium chloride.

Chemical Alteration of the Protein.

The question as to whether the hemagglutinating property is dependent upon the integrity of the protein has been approached by a study of the effects of chemical changes upon the latter.

Heat Coagulation.—The preparation studied could be denatured by heat when in solution. Below 68° the protein did not separate from aqueous solution. With slow heating, coagulation would

begin at 68°, but the greater part of the coagulable protein separated out between 75° and 81°. The filtrate from the coagulum at the higher temperature, showed a greatly lowered potency to agglutinate rabbit erythrocytes. At 100° coagulation became so complete that no agglutinating power remained. Heating the dry preparation to the same temperature was without influence upon solubility or potency of the material unless the heating

TABLE VI.

Influence of Peptic Digestion upon Activity of Hemagglutinating Protein.

	Minimum effective quantity of hemagglu- tinin.
	mg.
Untreated protein.....	0.0008
Dissolved in pepsin-HCl and neutralized at once.....	0.0008
	0.0007
After 1 hr. digestion.....	0.002
“ 1½ hrs. “	0.004
“ 3 days “	0.007
“ 6 “ “	Activity lost.

To 0.1 gm. portions of the protein weighed out exactly and placed in test-tubes were added 2 cc. of 0.1 N HCl solution and 2 cc. of 1 per cent pepsin solution with 2 cc. of water. The tubes were stoppered and incubated in a constant temperature water bath at 37.5° for the periods of time indicated in the summary. At the end of the incubation period, the solution was exactly neutralized by adding the calculated quantity of sodium hydroxide. The sodium chloride content was increased to make the solution exactly isotonic to rabbit erythrocytes after dilution to 20 cc. with water. Further dilutions were made with isotonic sodium chloride and the agglutinating potency of the preparations with rabbit erythrocytes were determined by the method already outlined. The minimum effective weight of hemagglutinin was expressed in terms of dry weight of the protein before digestion.

process was continued for a number of hours. Then the change was only slight. After 4 days of heating at 100° the agglutinating potency was lowered to one-thirtieth that of the original protein. Wienhaus has reported a similar finding with his phasin; heating the dry preparation for 2½ hours at 130° caused only a partial destruction of potency.

Acid Hydrolysis.—Short exposures to weak acid produced no

apparent effect upon the protein. When 0.1 gm. portions were treated with 1 cc. of 0.1 N hydrochloric acid and neutralized within 5 minutes, solubility and agglutinating potency were unaffected. Upon longer exposures, the protein gradually separated out in an insoluble form with an accompanying loss in agglutinating value. Thus after 3 days exposure to 0.1 N hydrochloric acid, the requisite weight of protein for clumping a given volume of erythrocyte suspension was increased to 5 times the amount required before the exposure to acid.

Peptic Digestion.—This also destroys the agglutinating potency. Hemagglutinating protein was dissolved in pepsin-HCl. When the solution was immediately neutralized no change in the minimum effective hemagglutinating dose of the protein could be detected. With the progress of digestion, however, the agglutinating activity of the solution was lowered; and at the end of a considerable period of incubation, it seemed to be entirely lost. The preceding summary of protocols (Table VI) illustrates these observations.

Mode of Action of Hemagglutinin.

There are indications that the hemagglutinins of plant origin resemble those of the serum. Comparable reactions appear to affect both groups of substances similarly. Both serum and plant hemagglutinins require electrolyte for their action; in both cases, a "fixation" reaction occurs between the agglutinin and the corpuscle prior to the flocculation which is dependent upon some physicochemical action of salts. By referring to Table V, the reader will note that rabbit erythrocytes may be washed with sucrose solution after exposure to hemagglutinin and still retain enough of the "fixed" material to clump upon addition of sodium chloride. The electrolyte is needed to complete the reaction. Wells (1925) has spoken of agglutination as a process of "salting out" in which electrolytes are of great importance. He points out further that one might think of the antigen-antibody (or agglutinin-corpuscle) reaction as resulting in the formation of an electrically amphoteric, colloidal suspension. The ions of electrolyte, then, cause precipitation by discharging the particles unequally. Evidence bearing upon this theory has been given by the work of Coulter (1920) who determined the isoelectric point

TABLE VII.

Variations in Amount of Hemagglutinin Fixed by Corpuscles.

Date of experiment.	Kind of erythrocyte.	Corpuscle suspension.	Minimum effective quantity of agglutinin:		
			For 1 portion of corpuscles.	For 2 successive portions.	For 3 successive portions.
		<i>vol. per cent</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Mar. 15	Rabbit.	2.5	0.0006	0.006	0.008
“ 18			0.0006	0.006	0.006
“ 24			0.0006	0.002*	
Apr. 9		5.0	0.0006	0.006	0.008
“ 13			0.0005	0.006	0.01
Mar. 12	0.0007		0.009	0.01	
Apr. 3	0.0005		0.003	0.003	
“ 12	0.0008		0.006	0.01	
Mar. 25	Man.	10.0	0.0009	0.003	
“ 18		20.0	0.005	0.008	
“ 28		2.5	0.0007	0.007	
Apr. 13		5.0	0.002	0.02	0.05†
Mar. 22	Chicken.	2.5	0.006	0.01	
Apr. 17			0.006	0.03	0.08
			0.006	0.04	
Mar. 20	Duck.	2.5	0.0006	0.006	0.006
		20.0	0.005	0.03‡	
Apr. 15	Dog.	2.5	0.0008	0.006	0.009
			0.0008	0.007	
		5.0	0.003	0.009	0.03†

To 0.5 cc. portions of erythrocytes of suspensions indicated in isotonic sodium chloride were added increasing quantities of hemagglutinin; and the volume was made up to 1 cc. with isotonic sodium chloride. After the corpuscles had clumped and sedimented, 0.5 cc. of the supernatant medium was removed and tested for agglutinating potency with a fresh suspension of corpuscles. Thus the amount of protein required in the initial dose for clumping two successive and three successive portions of corpuscles was determined.

* This value was obtained by using a 1.25 per cent suspension of corpuscles.

† This value was obtained by using a 2.5 per cent suspension of corpuscles.

‡ This value was obtained by using a 10.0 per cent suspension of corpuscles.

for normal sheep erythrocytes and showed that by the process of sensitization, the isoelectric point was moved in the direction of neutrality. Thus flocculation was made possible nearer neutrality, since particles in suspension are most easily aggregated and sedimented at their isoelectric points.

The two types of agglutinin also have similar fixation processes. According to the well established rule of Eisenberg and Volk (foreseen by Bordet) "for the same mass of cells, the absolute quantity fixed is directly proportional to the concentration of antibody; the relative quantity inversely" (Wells, 1925). In this connection, we found it interesting to calculate from data collected the maximum quantity of our agglutinin held by the cells of a given volume of suspension and to compare this with the minimum effective quantity of agglutinin for this same cell mass. With a uniform suspension of corpuscles in a series of tubes, the quantity of agglutinin was varied from the minimum requirement upward. By the standard procedure, not until 0.006 mg. or 10 times the minimum effective concentration had been used was there sufficient agglutinin free in the supernatant medium to cause clumping of a second unit of corpuscles (see Table VII). A liberal allowance for incomplete removal of unbound agglutinin being made, the bound fraction was at least 8 times the minimum required for clumping the mass of cells used. Corpuscles, then, do not combine with one and only one given quantity of a plant hemagglutinin. The amount "fixed" depends upon the quantity available in the medium.

Chemical Nature of Hemagglutinin.

The interpretation given by Eisler and Portheim (1926) to results which they obtained from a study of the hemagglutinin of *Phaseolus multiflorus* was that the active substance was not itself protein but was adsorbed onto the albumin fraction of the bean. They compared the increase in agglutinating value of the maturing seed with the increase in quantity of the various protein fractions and found that the former greatly exceeded the increase in total protein or in the albumin fraction itself (which contained agglutinin in higher concentration than any of the other fractions). In contrast to this conclusion, all the evidence afforded by the work reported here points toward the protein nature of the

hemagglutinin. We observed that the various phases of the denaturation process affecting the albumin preparation were accompanied by diminution of the agglutinating potency. Thus, the slow, denaturing effect of acids would in time destroy the agglutinin as completely as would the rapid process of heat coagulation. Neither was it possible to digest the protein by enzyme action without a concomitant loss of agglutinating potency. One would not necessarily expect this to happen were the agglutinin merely adsorbed onto the protein since it might then be liberated and be made available through the process of digestion. Peptic digestion changed the effectiveness of the agglutinin along with the clastic influence upon the protein molecule. By a peptic hydrolysis of 6 days duration, the agglutinin was rendered inert. Since from proteins with an agglutinating quality an individual hemagglutinating fraction has never been separated, and since chemical changes in the protein itself are accompanied by changes in the agglutinating potency, there is no reason for assuming two closely associated entities rather than one endowed with an unusual property. Moreover, the protein itself is reactive in such low concentration that if the agglutinin were a separate entity occurring as a contaminant, its effective concentration would be astonishingly minute.

Practical Considerations.

The outcome of this study suggests that hemagglutinating preparations of the sort that we have investigated may have a larger field of usefulness in the preparation of therapeutic serums than has hitherto been accorded to them. It should prove comparatively simple and inexpensive to prepare in adequate quantities readily soluble specimens of the bean albumin that can be duly sterilized and preserved in dry form for long periods ready for instantaneous use. The stoichiometric relations studied indicate that the quantities required for corpuscular agglutination are comparatively small. Furthermore, through preliminary tests on the species of blood to be agglutinated, it should be possible to estimate for large lots the dosage so selected that an excess of unfixed hemagglutinating protein will not remain in the serum. Danger of anaphylactic reactions through the retention of a

foreign protein like the bean albumin in a therapeutic serum ought thus to be successfully avoided.

SUMMARY.

A non-toxic, highly potent, soluble, hemagglutinating protein having the characteristics of an albumin was prepared in dry form from navy beans (*Phaseolus communis*). A quantitative, macroscopic method for measuring hemagglutination was devised and used for studying the variables affecting the reaction. Indispensability of electrolytes and the inhibiting influence of certain proteins, notably those of egg albumin and the serum proteins were demonstrated. Chemical changes in the protein which led to denaturation or hydrolytic cleavage were shown to be accompanied by a lessened hemagglutinative potency. The mode of action, the chemical nature, and some practical aspects relating to the application of the procedures to the production of therapeutic serums are discussed.

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THE COPPER CONTENT OF PLANT AND ANIMAL FOODS.*

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In a recent paper (1) the importance of a complete assay of copper in food materials was emphasized and an accurate quantitative method for the determination of this element in biological materials was outlined. In this paper the copper content of about 160 of our common food materials will be presented.

The information pertaining to the distribution of copper in foods is extremely meager. The most numerous data on the subject are those supplied by Guerithault (2) of France, by Quarataroli (3) of Italy, and by McHargue (4) of this country.

The data presented here have an added interest because the analyses were made on samples which had previously been analyzed for iron (5, 6) and manganese (7). A comparison of the variations of all three elements in the same samples of food materials is thus possible.

EXPERIMENTAL.

The preparation of the samples for analysis has already been described in detail (6) and the procedure need not be recounted here. The method used for the determination of copper was the modified Biazzo method as outlined by Elvehjem and Lindow (1).

In Table I are given the moisture and copper content, calculated both on the dry basis and the fresh basis, of 158 common food

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TABLE I.
Copper Content of Foods (Edible Portion).

Food.*	Moisture.	Copper content.		Food.*	Moisture.	Copper content.	
		Dry basis (100%).	Fresh material.			Dry basis (100%).	Fresh material.
	per cent	mg. per kg.	mg. per kg.		per cent	mg. per kg.	mg. per kg.
Almonds.....	3.9	12.6	12.1	Carrots.....	90.1	8.1	0.8
Apples, greening...	82.5	4.6	0.8	Cauliflower.....	91.4	16.5	1.4
" snow.....	83.9	7.5	1.2	Celery.....	94.0	2.0	0.1
Apricots, dried....	40.7	6.2	3.7	Celery cabbage....	94.3	10.4	0.6
Artichoke.....	84.4	20.1	3.1	Chard.....	91.5	13.2	1.1
Asparagus.....	91.8	17.2	1.4	Cheese, American..	32.3	2.6	1.8
Bananas.....	75.4	8.5	2.1	" Swiss.....	33.2	2.0	1.3
Beans, kidney.....	12.4	7.4	6.5	Cherries, red.....	88.0	11.7	1.4
" Lima.....	12.3	9.8	8.6	Chestnuts, Italian..	34.5	9.2	6.0
" navy (2)....	14.2	8.0	6.9	Chocolate, bitter...	1.9	27.2	26.7
" string.....	91.4	12.0	1.0	Cocoa.....	4.5	35.0	33.4
Beef, brains.....	82.6	12.0	2.1	Coconut.....	39.3	11.4	6.9
" casings.....	81.1	8.4	1.6	Corn, sweet,			
" kidney.....	81.1	6.0	1.1	bantam.....	89.1	5.9	0.6
" liver (5)....	71.6	75.7	21.5	Corn, sweet,			
" lung.....	80.3	11.4	2.2	evergreen.....	84.3	6.8	1.1
" pancreas....	80.0	4.0	0.8	Corn Flakes	6.2	2.0	1.9
" spleen.....	76.8	6.0	1.4	Corn-meal, white,			
Beefsteak, round..	75.1	3.0	0.8	prepared.....	6.2	2.0	1.9
" T-bone....	74.0	4.7	1.2	Corn-meal, yellow,			
Beet greens, tops...	90.3	9.3	0.9	prepared.....	6.0	2.1	2.0
" " roots.	87.2	7.7	1.0	Cranberries.....	88.5	7.8	0.9
Beets (2).....	83.5	11.5	1.9	Cream of Wheat....	7.4	3.1	2.9
Blackberries.....	84.1	10.0	1.6	Cucumbers.....	96.8	17.8	0.6
Blackcaps.....	82.8	8.0	1.4	Currants.....	32.7	16.6	11.2
Blueberries.....	81.3	6.0	1.1	Dandelion greens...	88.3	13.1	1.5
Bran Flakes.....	6.5	6.2	5.8	Dates, dried,.....	27.5	5.3	3.8
Brazil nuts.....	6.0	14.8	13.9	Eggs.....	71.9	8.2	2.3
Bread, white.....	35.0	5.2	3.4	Egg yolk.....	49.5	8.0	4.0
Brussels sprouts...	87.4	8.2	1.0	Eggplant.....	92.5	13.6	1.0
Butternuts.....	3.0	12.1	11.7	Figs, dried.....	38.0	5.7	3.5
Cabbage.....	92.6	6.8	0.5	Fish and sea foods.			
Calf, brains.....	76.8	7.5	1.8	Bass.....	77.0	6.2	1.4
" liver (6)....	73.2	164.4	44.1	Bluefish.....	76.7	10.0	2.3
Cantaloupe.....	90.5	6.1	0.6	Catfish.....	80.0	8.4	1.7

* When more than one sample was analyzed, the number of samples is indicated by the figure in parentheses.

TABLE I—Continued.

Food.*	Moisture.	Copper content.		Food.*	Moisture.	Copper content.	
		Dry basis (100%).	Fresh material.			Dry basis (100%).	Fresh material.
	per cent	mg. per kg.	mg. per kg.		per cent	mg. per kg.	mg. per kg.
Fish and sea foods				Lettuce, leaf (2)...	94.4	11.3	0.6
—Continued.				Milk...	87.5	1.2	0.15
Codfish.....	81.7	29.8	5.5	Molasses.....	26.2	26.2	19.3
Flounder.....	80.0	7.3	1.5	Mushrooms.....	71.2	61.7	17.9
Haddock.....	78.8	13.4	2.8	Muskmelon, honey			
Halibut.....	67.3	7.1	2.3	dew.....	89.4	6.5	0.7
Herring.....	77.6	11.1	2.5	Oatmeal.....	6.4	5.4	5.0
Lobster.....	81.1	38.8	7.3	Olives.....	77.0	14.7	3.4
Mackerel.....	77.6	15.4	3.4	Onions.....	93.7	13.4	0.8
Oyster (2).....	87.5	245.8	30.7	Oranges.....	87.6	6.4	0.8
Perch.....	80.4	18.7	3.7	Oyster plant.....	76.6	11.4	2.7
Pickrel.....	72.5	12.3	3.4	Parsley.....	87.6	17.3	2.1
Pike.....	80.2	8.5	1.7	Parsnips.....	82.7	7.0	1.2
Red snapper....	79.2	7.6	1.6	Peaches, dried....	37.4	6.3	2.7
Salmon.....	75.7	7.8	1.9	Peanuts.....	2.0	9.7	9.6
Scallops.....	81.3	12.3	2.3	Pears.....	83.9	6.3	1.0
Shad.....	69.8	7.7	2.3	Peas, green.....	75.2	9.8	2.4
Shrimp.....	70.4	14.4	4.3	" split.....	9.5	15.5	14.0
Trout, lake.....	70.9	10.3	3.3	Pecans.....	2.3	13.9	13.6
Whitefish.....	79.8	9.7	1.9	Peppers, green....	94.0	16.1	1.0
Flour, buckwheat..	9.0	7.7	7.0	Pineapple.....	92.0	8.3	0.7
" Graham.....	6.5	5.2	4.9	Pistachio nuts.....	4.0	12.2	11.7
" patent.....	8.9	1.9	1.7	Plums, blue.....	84.9	9.7	1.5
" rye.....	6.4	4.4	4.2	Pork chops.....	54.4	6.8	3.1
Gooseberries....	90.1	8.1	0.8	Potatoes.....	78.2	8.0	1.7
Grapes, Malaga....	79.6	4.8	0.9	Potatoes, sweet....	72.1	5.2	1.5
Grapefruit.....	92.8	4.8	0.3	Poultry.			
Grape juice.....	96.0	5.3	0.2	Chicken, dark			
Hazelnuts.....	3.8	14.0	13.5	meat.....	67.5	12.7	4.1
Hickory nuts.....	2.9	14.7	14.3	Chicken, white			
Hog, liver (5)....	68.7	20.8	6.5	meat.....	76.6	11.5	2.7
Hominy.....	7.5	2.0	1.9	Duck.....	43.7	7.3	4.1
Honey.....	18.2	2.5	2.0	Goose.....	57.0	7.7	3.3
Kohlrabi.....	90.7	15.0	1.4	Turkey, dark			
Kumquats.....	85.0	5.5	0.8	meat.....	72.1	7.3	2.0
Lamb chops.....	54.2	9.1	4.2	Turkey, white			
Lemon.....	96.0	10.2	0.4	meat.....	72.2	5.4	1.5
Lettuce, head.....	96.6	11.6	0.4	Prunes, dried.....	44.1	7.3	4.1

TABLE I—*Concluded.*

Food.*	Mois- ture.	Copper content.		Food.*	Mois- ture.	Copper content.	
		Dry basis (100%).	Fresh mate- rial.			Dry basis (100%).	Fresh mate- rial.
	per cent	mg. per kg.	mg per kg.		per cent	mg. per kg.	mg. per kg.
Puffed Rice.....	10.9	6.3	5.6	Spinach.....	81.9	6.9	1.2
Puffed Wheat.....	8.6	7.6	7.0	Squash, Hubbard..	90.4	4.2	0.4
Pumpkin.....	91.7	4.0	0.3	Strawberries.....	90.3	1.9	0.2
Quince.....	82.5	7.8	1.4	Tangerines.....	86.0	6.2	0.9
Radishes.....	94.4	28.7	1.6	Tomatoes (2).....	94.2	9.9	0.6
Raisins, seeded....	28.2	3.8	2.7	Turnips.....	91.5	11.0	0.9
“ seedless....	31.9	3.0	2.0	Veal chops.....	72.6	9.1	2.5
Raspberries, red...	84.1	8.3	1.3	Walnuts, English...	3.3	10.3	10.0
Rhubarb.....	94.4	9.5	0.5	Watercress.....	92.5	5.3	0.4
Rice, polished.....	9.5	2.1	1.9	Watermelon.....	92.7	9.1	0.7
“ unpolished...	9.5	4.0	3.6	Wheat bran.....	3.3	12.1	11.7
Rutabagas.....	80.9	8.0	1.5	“ germ.....	10.4	14.2	12.7
Shredded Wheat...	8.1	6.7	6.2				

materials. The figures range from 44.1 mg. of copper per kilo of fresh calf liver to 0.1 mg. of copper per kilo of fresh celery. Within these limits the various groups of foodstuffs in order of their average copper content per kilo of fresh material come as follows: ten nuts, 11.6 mg.; four dried legumes, 9.0 mg.; nineteen cereals, 4.7 mg.; eight dried fruits, 4.2 mg.; four kinds of poultry, 3.0 mg.; seventeen kinds of fish, 2.5 mg.; thirteen animal tissues, 1.7 mg.; two green legumes, 1.7 mg.; eleven roots, tubers, stalks, and bulbs, 1.4 mg.; fourteen leafy vegetables, 1.2 mg.; twenty-seven fresh fruits, 1.0 mg.; ten non-leafy vegetables, 0.7 mg. The first four groups owe their high rank largely to the low percentage of moisture contained in these foods. In contrast to the conspicuous place which they occupied as based on their iron content (6), the green leafy vegetables come far down the series. It is rather unexpected to find them surpassed in copper content by roots, tubers, stalks, and bulbs.

A few individual foods are strikingly high in copper. Besides calf liver, which has already been mentioned, oysters, chocolate, cocoa, and molasses are conspicuously high in this element.

The same organ from animals of different age or species shows

marked differences in copper content. Calf liver contains 2 times as much copper as beef liver, and the latter contains 3 times as much copper as hog liver. Because of these wide variations the figures for liver were omitted in calculating the average for animal tissue.

TABLE II.

Degree of Variation in Iron, Manganese, and Copper Content of Different Classes of Food Materials.

Class.	No. of samples.	Average.	Minimum.	Maximum.
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Iron in fresh material.

		mg. per kg.	mg. per kg.	mg per kg.
Fresh fruits.....	23	6.6	2.3 Watermelon.	22.8 Grapes.
Nuts.	12	41.0	21.4 Walnut.	79.2 Pistachio nut.
Roots and tubers...	14	11.0	3.0 Onion.	23.6 Beets.
Vegetables, leafy...	7	69.0	3.4 Cabbage.	192.1 Parsley.

Manganese in fresh material.

Fresh fruits.....	13	4.0	0.2 Watermelon.	22.9 Blueberries.
Nuts.....	3	13.3	6.3 Pistachio.	18.0 Walnut.
Roots and tubers...	7	3.2	0.5 Onion.	13.5 Beets.
Vegetables, leafy...	8	6.6	0.8 Cabbage.	12.6 Beet greens, tops.

Copper in fresh material.

Fresh fruits.....	27	1.0	0.2 Strawberries.	3.4 Olives.
Nuts.....	10	11.6	6.0 Chestnuts.	14.3 Hickory nuts.
Roots and tubers..	11	1.4	0.8 Carrots.	2.7 Oyster plant.
Vegetables, leafy..	14	1.2	0.4 Watercress.	3.1 Artichoke.

Unlike the data obtained for the iron content of salt water and fresh water fish (6), the average figures for the copper content of the two groups are practically the same.

One of the outstanding features of this series of analyses is the low order of variation among samples of food materials that fall in the same class. This fact becomes still more apparent when

the variations are compared with those of iron and manganese in the same samples. In Table II figures are given for the variations in iron, manganese, and copper of four different classes of foods. In the class of leafy vegetables the iron content of the highest is almost 60 times that of the lowest member. The maximum figure for manganese is about 15 times the minimum, while the maximum figure for copper is only 8 times the minimum.

Because of the limited data available in the literature, it is difficult to compare the results obtained by different workers. However, it is worth noting that our figures compare favorably with those of Guerithault (2) and Quartaroli (3) who have worked on a different continent and who have used different methods. In some instances there is a wide variation, but the order of the series is not generally disturbed. The copper content of twenty-seven foods analyzed by Guerithault averaged 3.6 mg. per kilo of fresh material, while the copper of the same materials analyzed by us averaged 3.1 mg. per kilo. A similar comparison gave an average of 14.1 mg. of copper per kilo of dry matter for eighteen samples analyzed by Quartaroli and 10.4 mg. per kilo for the same group reported here.

SUMMARY.

The copper content of about 160 samples of our common food materials has been determined. The figures range from 0.1 mg. of copper per kilo of fresh celery to 44.1 mg. per kilo of fresh calf liver. The classes of foods in descending order of copper content are as follows: nuts, dried legumes, cereals, dried fruits, poultry, fish, animal tissues, green legumes, roots, etc., leafy vegetables, fresh fruits, and non-leafy vegetables. The copper content of leafy vegetables does not place them in the preeminent position that they hold with reference to their iron content.

A wide variation was found in the copper content of livers from different animals; calf liver was highest and hog liver was lowest.

The copper content of oysters proved to be strikingly high and surpassed all the sea foods in this element.

The degree of variation in the copper content of foods falling in the same class was less than that of either manganese or iron.

There is a wide distribution of copper in food materials, no

food examined being without this element. Certain milled cereals, such as polished rice and patent wheat flour, are very low in copper as compared to the whole grain from which they were made.

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THE COPPER CONTENT OF FEEDINGSTUFFS.*

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INTRODUCTION.

The clearly demonstrated rôle of copper as a supplement to iron for hemoglobin building in the rat (1) will undoubtedly lead to further studies on the importance of this element in the nutrition of domestic animals. Before this work can progress to best advantage, we need to know the distribution of copper in the common feedingstuffs. The literature on the copper content of this type of material is even more limited than that for human foods, the paucity of which was emphasized in a previous paper (2). Quartaroli (3) and McHargue (4) have made some analyses to determine the content of some of the rarer elements in feeds, the former presenting data for copper and manganese content and the latter for copper, manganese, iron, and zinc.

In a previous paper (5), the manganese and iron content of 51 feeds was given. Since these samples were still available, it was thought desirable to analyze this same series for copper, which would enable us to compare the relative abundance and distribution of these three elements in the same feeds.

In view of the fact that feeds grown on different soils may vary widely in content of the rarer elements, we decided to determine if fertilization with copper salts would influence the copper content of the crops grown. It is valuable to know how much the copper content of the soil can fluctuate before any change can be detected in the copper content of the crop grown on that soil.

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EXPERIMENTAL.

The feeds used in this study were those prepared according to the method presented in a paper by Skinner and Peterson (5).

TABLE I.
Copper Content of Feedingstuffs.

Material.	Copper in dry matter (100°).	Material.	Copper in dry matter (100°).
	<i>mg. per kg.</i>		<i>mg. per kg.</i>
Alfalfa hay.....	9.1	Linseed oil meal.....	21.8
“ “ first cutting (Wisconsin).....	4.5	Millet, Japanese.....	9.1
Alfalfa hay, second cutting (Wisconsin).....	14.8	Oats.....	6.8
Alfalfa hay, first cutting (Colorado, Sample 1).....	6.9	Oat groats.....	7.6
Alfalfa hay, first cutting (Colorado, Sample 2).....	11.9	“ straw.....	3.8
Barley.....	7.2	Rape.....	7.7
Blood meal.....	13.1	Red clover hay.....	17.6
Blue-grass.....	8.3	Rye.....	5.9
Brewers' dried grain.....	16.4	“ straw.....	4.4
Buckwheat feed.....	13.7	Sorghum fodder.....	8.7
Coconut oil meal.....	10.5	Soy beans.....	22.7
Corn, white.....	4.4	“ bean hay.....	8.2
“ yellow.....	4.7	Sugar beets.....	8.5
“ gluten meal.....	35.1	“ beet tops.....	6.4
“ oil cake meal.....	13.2	Sweet clover hay.....	11.8
“ silage.....	3.8	Tankage.....	23.1
“ stover.....	4.0	Timothy hay.....	2.2
Cottonseed meal.....	21.8	Tobacco.....	16.8
Distillers' grain.....	38.4	Vetch hay.....	9.6
Emmer.....	9.6	Wheat.....	7.8
Gluten feed.....	89.5	“ bran.....	16.4
Kaffir, white.....	8.0	“ germ.....	9.0
		“ gluten.....	17.2
		“ middlings, flour.....	12.1
		“ straw.....	2.1

The Biazzo method as modified by Elvehjem and Lindow (6) was used in making all of the copper determinations.

A garden plot 15 by 160 feet in size was set aside for the fertilization experiment. The soil of the whole area was of uniform texture and fertility. One-half was used in the original state of fertility

for the control, while the other portion was treated at the rate of 50 pounds of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per acre. The salt was added in solution so as to insure uniform distribution over the soil. If McHargue's figure (7) of 7.2 parts of copper per million of soil is at all representative of the average copper content of soils, then our treatment would have doubled the copper in the fertilized plot.

In both the control and the treated areas oats, lettuce, carrots, tomatoes, navy beans, and kidney beans were grown and harvested

TABLE II.
Copper Content of Crops Grown on Soils Containing Different Amounts of Copper.

Material.	Copper per kilo dry matter.		Increase.
	No copper addition.	Copper added 50 lbs. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per acre.	
	mg.	mg.	per cent
Lettuce 1.....	11.7	14.7	25.6
“ 2.....	12.1	15.2	25.6
Tomatoes, green....	9.3	10.4	11.8
“ ripe.....	11.2	12.6	12.5
Beans, navy.....	7.4	9.5	28.4
“ kidney.....	7.4	9.5	28.4
Carrots.....	8.1	8.9	9.8
Oat, grass.....	7.9	10.1	27.8
“ seed.....	6.5	7.5	15.4
Lettuce*.....	12.1	30.1	148.0

* Copper added equivalent to 500 pounds of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per acre.

under conditions which were identical with the exception of the copper content of the soil. Especial precautions were taken in the harvesting of the crops to prevent any copper contamination due to adhering soil particles. The crops were sampled, dried, and analyzed in the usual manner.

DISCUSSION.

The copper content of the forty-seven feeds analyzed is given in Table I. The average copper content of forty-two of the feeds analyzed was 13.5 mg. per kilo of dry matter. The average iron and manganese contents of the same feeds, calculated from the

data presented in a previous publication (5), are 199.0 and 65.8 mg. per kilo of dry matter, respectively. Thus the copper content of feedingstuffs is about one-fifth of the manganese, and one-fifteenth of the iron content.

Some of the manufactured feeds are unusually high in copper; *e.g.*, gluten feed, corn gluten meal, and distillers' grain rank among the highest in the group. The high copper content of such feeds is probably due to copper contamination during the process of manufacture.

The variation in the copper content of the majority of the feeds is very small. The straws and stovers are the lowest, hays and grasses are intermediate, and the seeds and seed products are highest in copper.

In Table II the copper content of the crops grown on the fertilized and untreated soil is given together with the percentage increase of copper in the crops produced on the treated plot. The figures show that there is a small though definite increase in the copper content of the crops grown on the plot treated with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at the rate of 50 pounds per acre.

In a single experiment, involving only lettuce, the copper fertilization was increased tenfold; *i.e.*, the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ added was equivalent to 500 pounds per acre. In this case the increase in the copper content of the lettuce grown on the copper-treated plot over that produced on the untreated soil was 148 per cent. These data, though limited, indicate that the copper content of a crop can be increased within certain limits by fertilization of the soil with a copper salt.

SUMMARY.

The copper content of forty-seven common feeds is given. The average copper content is one-fifth of the manganese, and one-fifteenth of the iron in forty-two of these feeds.

Certain manufactured feeds are unusually high in copper. This is probably due to contamination.

The feedingstuffs arranged in ascending order of copper content are as follows: straws and stovers, hays and grasses, and seeds and seed products.

The copper content of the crop can be increased within certain limits by fertilization of the soil with a copper salt.

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TRYPTOPHANE AND GROWTH.

I. GROWTH UPON A TRYPTOPHANE-DEFICIENT BASAL DIET SUPPLEMENTED AT VARYING INTERVALS BY THE SEPARATE FEEDING OF TRYPTOPHANE.

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It is a common practice in certain types of investigations to supplement a deficient diet by feeding a single component apart from the basal ration. Such a procedure has the advantage of insuring the ingestion from day to day of a constant quantity of the supplementing agent. In the case of the vitamins, for which there appears to be a storage capacity in the organism, separate feeding seems to afford just as satisfactory growth as when the accessory substances are incorporated in the food. On the other hand, it may be questioned whether the single feeding of an amino acid to an animal on an otherwise adequate diet is capable of inducing as satisfactory growth as when the substance in question is administered more frequently. Apparently, amino acids ingested in excess of the anabolic needs are not retained. Thus the administration at one time of an amount necessary to meet the daily needs of the organism might result in the utilization for growth purposes of only a small portion, while the larger quantity might be disposed of by oxidation and excretion. If such were the case the animal would experience a deficiency of the amino acid during at least a portion of each 24 hours.

The above considerations led us to conduct the following experiments in which young rats were fed a tryptophane-deficient basal

* The experimental data in this paper are taken from a thesis submitted by Clarence P. Berg in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

diet supplemented at intervals of 6, 12, 24, or 48 hours by the separate feeding of appropriate quantities of tryptophane. The basal diet was composed of acid-hydrolyzed casein 14.7, cystine 0.3, dextrin 40, sucrose 15, lard 19, cod liver oil 5, salt mixture¹ 4, and agar 2 per cent. The hydrolyzed protein was prepared as follows: 1 kilo of casein was refluxed on a sand bath for 16 hours with a mixture of 1250 cc. of concentrated sulfuric acid and 3750 cc. of distilled water. The resulting solution was diluted with about 3 volumes of distilled water, and treated with finely powdered barium hydroxide until only a trace of sulfuric acid remained. After filtering off the barium sulfate, the precipitate was washed twice by suspending in approximately 5 liter portions of distilled water and passing in steam until the suspension had been thoroughly heated. The combined filtrate and washings were evaporated *in vacuo* to a volume of approximately 10 liters, and the remaining trace of sulfuric acid was removed by the cautious addition of a saturated solution of barium hydroxide. After filtration, the solution was evaporated to dryness, first by means of a vacuum still, and finally in a vacuum oven. The resulting material was ground and passed through a 40 mesh sieve. The yields in several runs varied from 700 to 850 gm. Vitamin B was supplied in the form of pills composed of 75 mg. of yeast, 30 mg. of dextrin, and sufficient water to make a stiff dough. The pills were administered to all animals at 6 hour intervals. Thus each rat received a total of 300 mg. of yeast in the course of each 24 hours.

Sixteen rats from two litters were divided into four equal groups. The first group received 40 mg. of tryptophane at the same hour every other day; the second, 20 mg. daily; the third, 10 mg. at intervals of 12 hours; and the fourth, 5 mg. at intervals of 6 hours. Thus the tryptophane given to each rat averaged 20 mg. daily. This was incorporated in the vitamin pills at the times indicated. The only variable between the groups was the *frequency* with which the amino acid was administered. An abundant supply of the basal diet was kept before the rats at all times.

Inasmuch as one of the four pills which each animal received daily had to be given during the early morning hours, it became desirable to devise a mechanical feeder capable of introducing the

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

accessory agents into the cages at the proper times without necessitating the presence of an attendant. For this purpose the cages were placed in parallel rows of eight each. Between each pair of cages an iron stand was securely fastened to the table top by means of screws. Clamps attached to the stands held glass tubes 16 cm. long through which passed glass rods 28 cm. in length. The latter were of such a size that they fitted the tubes snugly and still could be rotated freely. At the ends of each rod just above the cages were fastened No. 3 rubber stoppers in the sides of which had been made small holes to serve as receptacles for the supple-

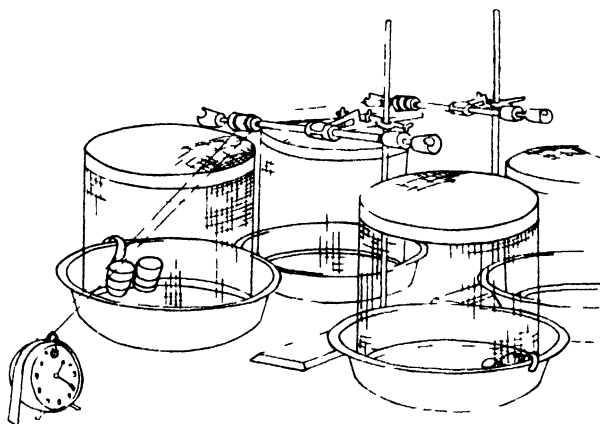


FIG. 1. Automatic feeder.

menting pills. A cork about 3 cm. in diameter on each rod was provided with two circular grooves, and served as a pulley. The motive power was furnished by an alarm clock fastened to the table by means of a metal band. The bell and alarm key were removed, and the latter was replaced by a small pulley. Waxed cords connected the pulley on the clock with the corks on the rotating rods. Thus at any desired time the release of the alarm rotated the glass rods and permitted the pills to drop through apertures in the cage tops. The essential features of the contrivance are illustrated in Fig. 1. Ordinarily, the pills were administered by hand at 8 a.m., 2 p.m. and 8 p.m. The automatic feeder was employed at 2 a.m. only.

It is perhaps worthy of note, as an interesting illustration of conditioned reflexes, that after the first few days the rats associated the buzzing of the alarm with the administration of the accessory substances. This fact was observed when the apparatus was being tested, as was done daily, to insure its successful operation. At the first sound of the alarm all the rats stood upon their hind feet and watched for the dropping of the pills. When

TABLE I.

Growth upon Tryptophane-Deficient Basal Diet When Tryptophane Is Fed Separately at Varying Intervals.

Rat No. and sex.	Litter.	Average daily food consumption	Initial weight.	Final weight	Gain.	Tryptophane administration.
		gm	gm.	gm.	gm.	
1028 ♀	A	5.1	58	108	50	40 mg. every 48 hrs.
1029 ♂		5.5	62	107	45	40 " " 48 "
1030 ♀		5.8	57	134	77	20 " " 24 "
1031 ♂		5.6	58	124	66	20 " " 24 "
1032 ♀		7.0	56	141	85	10 " " 12 "
1033 ♂		6.2	58	150	92	10 " " 12 "
1034 ♀		7.1	56	143	87	5 " " 6 "
1035 ♀		6.1	55	142	87	5 " " 6 "
1036 ♀	B	5.6	48	116	68	40 mg. every 48 hrs.
1037 ♂		5.2	48	116	68	40 " " 48 "
1038 ♀		6.4	44	123	79	20 " " 24 "
1039 ♂		5.8	47	137	90	20 " " 24 "
1040 ♀		6.6	44	146	102	10 " " 12 "
1041 ♂		6.6	46	146	100	10 " " 12 "
1042 ♀		6.5	40	148	108	5 " " 6 "
1043 ♀		7.0	38	150	112	5 " " 6 "

the latter failed to appear the animals diligently searched the bottoms of the cages for the missing dietary supplements. A similar reaction was noted each day.

The results of the experiments are summarized in Table I and Chart I. In the latter the lines connect points representing the average initial and average final weights of four rats. As will be observed, there is a progressive increase in total gain with increase in frequency of tryptophane administration up to and including

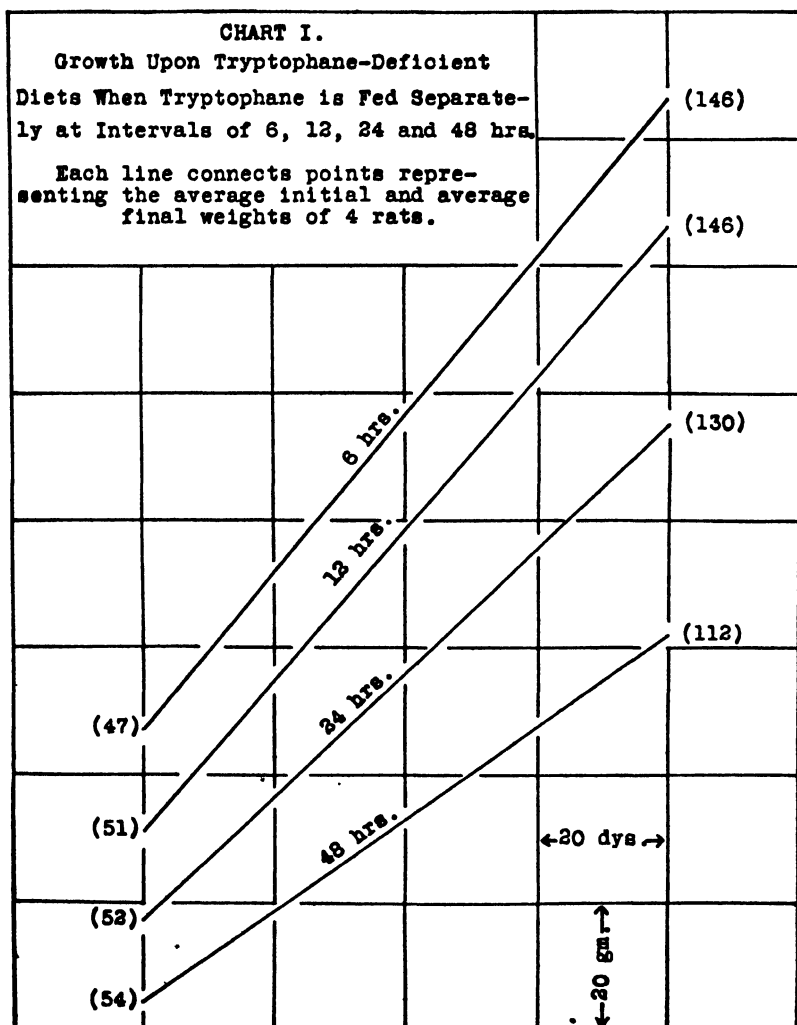


CHART I. The numbers in parentheses denote the initial and final weights of the rats.

the group of animals which received the amino acid at 12 hour intervals. Thus, those which were given tryptophane daily grew more rapidly than those which received a double dose every other day; and those which received half of the daily allowance at 12 hour intervals made greater gains than those which received a single dose each 24 hours. Feeding the amino acid more frequently than twice daily appeared to produce little if any further improvement. The latter was unexpected inasmuch as all of the animals grew at subnormal rates. In other experiments, which need not be presented in detail here, the inclusion of tryptophane in the basal ration led to much more rapid growth than when it was fed separately. In this respect the behavior of tryptophane appears to differ from that of certain other essential amino acids, notably histidine. With the latter, the acceleration in growth following separate feeding is almost if not quite as pronounced as that which results from its admixture with the other articles of food. Possibly amino acids vary in respect to the frequency with which they must be administered in order to provide optimum conditions for growth. But until specific information for each is obtained it appears advisable in studies involving their separate feeding to administer them at least twice daily.

SUMMARY.

The frequency of administration of tryptophane to animals upon tryptophane-deficient basal diets exerts a marked influence upon the rate of increase in body weight. Feeding half the daily allowance at intervals of 12 hours induces better growth than when the total day's allotment is administered at one time. More frequent administration than twice daily appears to exert little if any further benefit. It is suggested that amino acids may differ as to the frequency with which they must be supplied when fed apart from the other components of the ration; but until more definite information is secured it appears desirable in such studies to administer the supplementing compound at least twice daily.

THE METABOLISM OF SULFUR.

XV. THE RELATION OF THE PROTEIN AND CYSTINE CONTENT OF THE DIET TO THE GROWTH OF THE HAIR IN THE WHITE RAT.

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The use of laboratory animals as experimental subjects in studies in nutrition has led to suggestions, for the most part incidental, of a possible relationship of the protein content of the diet to the growth of the hair. Thus Abderhalden (1) noted that in one of his dogs, which had been clipped just prior to the beginning of the experiment, a full coat of hair was produced during the time in which the animal was fed a diet containing completely hydrolyzed protein as the sole source of nitrogen. McCollum, Simmonds, and Parsons (2) reported the loss of hair in white rats fed on diets containing a low percentage of protein, derived largely from potato. Boas (3) observed that the ingestion of diets in which a commercial preparation of egg white was the sole source of protein resulted in the loss of hair of white rats. This effect was not noted when fresh egg white was fed (4). It should be noted that improvement was obtained by the substitution of either 20 per cent casein or serum albumin for the proteins of the egg white. Hartwell (5) observed that loss of fur in white rats was closely correlated with the quality of the protein of the diet. However, since a favorable response was obtained with the addition to the diet of either the biologically adequate protein, casein, or the incomplete protein, gelatin, it is difficult to interpret these beneficial results as due solely to the variations in the amino acid make-up of the proteins.

That production of hair is related to the dietary intake of the

amino acid, cystine, which is present in notable amounts in hair and similar epidermal tissues (6), was suggested by Evvard, Dox, and Guernsey (7). The offspring of sows fed during pregnancy with "black albumen," a commercial protein concentrate derived from blood, were larger with heavier and darker coats of hair than the controls, whose mothers had received a diet derived from cereals chiefly. They suggested that since "a large proportion of the sulfur found in black albumen is supposedly present as cystine, it is not unreasonable to assume that the addition of black albumen furnishes the cystine, the basal constituent of hair growth."

In one rabbit on an experimental diet, the protein element of which was derived from wheat gluten and casein, Sjollesma (8) noted the appearance of bald spots, which upon the addition of 0.18 gm. of cystine to the ration daily, became covered with thick fur in 14 to 20 days, an effect which was "marvelous."

That cystine is importantly concerned in the growth of human hair has been maintained by Zuntz and coworkers (9), who used a commercial product (Humagsolan) prepared by the hydrolysis of keratin. The beneficial results in the increased growth of the hair were attributed to the presence of cystine in the material fed. Blaschko (10) was able to confirm the findings of Zuntz, the preparation being effective in cases of baldness, providing the papillæ had not been destroyed. Fuhs (11), however, was unable to obtain such marked beneficial results.

The data in support of the theory of the supposed relation of the protein or cystine of the food to the production of the hair have thus been derived from observations made incidentally during the conduct of other experiments or from studies in which the influence of the other possible variable factors has not been excluded. Factors of the diet, other than the protein or cystine content, have usually been varied, and there has been no satisfactory criterion of hair production or of a change in cystine metabolism.

It has been the purpose of the present series of experiments to study the production of hair in young white rats, when fed diets varying only in their protein content; *i.e.*, diets in which the amount of cystine has been shown to be the limiting factor. If in cases of an inadequate supply of cystine in the diet, the demand for cystine both for the growth of more essential tissues and also

for the elaboration of the less essential epidermal structure, the hair, cannot be met, will the demand for cystine for purposes of growth take precedence over the requirements for the production of hair?

This problem involves the more general question of the competition between a more essential and a less essential tissue for a unit necessary for each. The high content of the hair in cystine and the possibility of actually measuring the growth of this structure under varying conditions of nutrition make this tissue almost ideal for the study of this problem of competition for an essential chemical constituent.

EXPERIMENTAL.

White rats at the age of 30 days were selected from our colony for these studies in such a manner that litter mates were distributed between the several diets chosen, thus eliminating as far as possible variation between litters. Because of the difficulty in obtaining the necessary number of animals in a sufficiently short time to eliminate any possible seasonal differences, it was necessary to extend the period of the experiments over 6 months from May to November. Fourteen animals were placed in each of the experimental groups, nine of each group being killed at the end of 11 weeks. In order to determine whether white rats under laboratory conditions formed a winter coat of a character different from that formed in spring or summer, the remaining five animals of each group were continued on the experimental diets for an additional 6 weeks. As no clear evidence of the formation of such a coat was obtained, the two periods are not differentiated in the summary tables given later.

The diets chosen were based upon the experimental data of Sherman and Woods (12), which have been confirmed repeatedly in our own laboratory (13). These investigators were able to demonstrate that the growth of white rats on a basal diet whose protein was derived from whole milk powder (16.7 per cent) was limited by the cystine content of the diet and that improved rates of growth could be obtained in proportion to the amount of cystine or casein added as a supplement to the basal diet. The basal diet (Diet A) used in our experiments, together with the modifications designed to increase the cystine content through the

addition of varying amounts of casein, is presented in Table I. It will be noted that when casein was introduced into the diet, it replaced an equal amount of dextrin, the calorific value thus remaining approximately the same for all the diets.

As controls, four representative groups from our colony were used for the collection of hair. In order to secure information as to the hair production prior to the beginning of the experimental periods, one group (Group V) was killed at the age of 30 days and the amount of hair which had been produced at that age was determined. A second control group (Group VI) received an adequate mixed diet of ground biscuit (50 per cent), powdered whole milk (20 per cent), wheat germ (20 per cent), and casein

TABLE I.
Experimental Diets.

Diet.....	A	B	C	D	E
Whole milk powder.....	16.70	16.70	16.70	16.70	16.70
Dextrin.....	79.00	78.53	77.00	71.00	63.00
Cod liver oil.....	1.00	1.00	1.00	1.00	1.00
Salt mixture*.....	1.65	1.65	1.65	1.65	1.65
Dried yeast.....	1.65	1.65	1.65	1.65	1.65
Casein.....		0.47	2.00	8.00	16.00

*Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 557 (1919).

(10 per cent) during the same periods of time as the animals which were fed the diets in which the cystine content was the limiting factor. It was observed early in the course of the experiments that at the end of the 11 week period, the rats which received the least amount of casein and cystine (Group I, Diet B) had reached an average body weight of about 120 gm. As a further control on this group, a third series (Group VII) was fed the same stock diet as Group VI, fully adequate for normal growth, but was killed for a study of the amount of hair produced, when the animals had attained a body weight of approximately 120 gm. This stage in development was reached when the animals were from 6 to 7 weeks old; i.e., after a period of 2 to 3 weeks on the stock diet. A fourth control group (Group VIII) was composed of adult females varying in age from 12 to 14 months.

For the collection of the hair, the pelts were removed immediately after the sacrifice of the animals by cutting on a medial line of the ventral surface, about the legs at the tarsal bones, and about the neck on a line immediately posterior to the ears. It was thus possible to remove practically all of the hide except the small portions on the ears and fore part of the head and the extreme ends of the limbs. The pelts were immediately scraped free from as much subcutaneous tissue as possible and weighed while still moist. Each pelt was then immersed in a liter of phosphate solution buffered at a pH of 6.8 to which 1 gm. of a commercial pancreatin preparation was added. After incubation at 37° for 24 hours, the pelts were removed from the digestion mixture and the hair, loosened in the digestion, was separated from the other tissue. In order to remove adhering tissue, the hair was suspended repeatedly in distilled water, and the mixture was stirred and filtered through 60 mesh copper gauze. The hair was then transferred to weighed paper extraction thimbles; the thimbles were allowed to drain and then dried for 24 hours at 60° in a vacuum oven. The thimbles were then transferred to extraction apparatus of the Bailey-Walker type and the hair was extracted for periods of 24 hours each with absolute alcohol, ether, and distilled water. The thimbles were again dried *in vacuo* at 60° and stored in a desiccator over calcium chloride. For the weighing of the thimbles and hair, the thimbles were placed in thin glass-stoppered weighing bottles.

The animals were weighed weekly throughout the experiments. Body surface was calculated by the formula of Carman and Mitchell (14). From the data obtained were calculated the ratios of the weight of the hair to body weight and to body surface, which are expressed in Tables III to VII as mg. of hair per gm. of body weight or per sq. cm. of body surface.

DISCUSSION.

The average final weights of each group of animals are presented in Table II. It will be observed that, in confirmation of the work of Sherman and Woods (12), a significant change in the rate of growth occurred on the addition of increasing amounts of casein to the diet. Thus the animals of Group I, to whose diet a very small amount of casein (0.47 per cent) was added, attained

TABLE II.
Average Final Weight of Rats.

Group No.	Diet.	Age 15 wks. Weight.		Age 21 wks. Weight.		Group No.	Diet.	Weight.	
		Males.	Fe-males.	Males.	Fe-males.			Males.	Fe-males.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>			<i>gm.</i>	<i>gm.</i>
I	B	124.7	101.4	144.9	143.4	V	Stock.	57.7	63.1
II	C	161.4	147.9	203.5	155.3		(Rats 30 days old.)		
III	D	249.9	154.7	270.0	175.1	VII	Stock.	122.5	113.7
IV	E	270.5	183.5	297.7	178.0		(Rats 6 to 7 wks. old.)		
VI	Stock.	297.1	158.1	312.3	193.6	VIII	Stock. (Adults.)		229.3

TABLE III.
Group V.

Young rats, 30 days old.

Rat No.	Sex.	Weight.	Surface.	Hair.	Hair.	
					Per gm. body weight.	Per sq. cm. body surface.
		<i>gm.</i>	<i>sq. cm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>
93	F.	67.4	188.1	0.4000	5.93	2.12
96	"	61.5	177.0	0.3220	5.23	1.81
97	"	70.5	193.8	0.5876	8.33	3.03
101	"	53.2	160.6	0.3836	7.21	2.38
92	M.	56.2	166.6	0.2908	5.17	1.74
94	"	59.9	173.9	0.4122	6.88	2.37
95	"	51.8	157.8	0.2461	4.75	1.55
98	"	51.1	156.4	0.5108	9.99	3.26
99	"	62.5	178.8	0.4680	7.48	2.61
100	"	64.7	183.0	0.5220	8.06	2.85
Average.....					6.90	2.37

Rats killed at average weight of 120 gm.; age 6 to 7 weeks.

Rat No.	Sex.	Weight.	Surface.	Hair.	Hair.	
					Per gm. body weight.	Per sq. cm. body sur- face.
109	F.	119.4	275.4	1.7606	14.74	6.39
111	"	106.9	255.8	1.1391	10.65	4.45
112	"	109.4	259.8	1.2281	11.22	4.72
113	"	120.4	276.9	1.3289	11.03	4.79
116	"	115.3	269.1	1.2114	10.50	4.50
117	"	111.3	262.8	1.1989	10.77	4.56
110	M.	118.2	273.6	1.5002	12.69	5.48
114	"	127.4	287.6	1.6096	12.63	5.59
115	"	122.1	279.5	1.2340	10.10	4.41
Average					11.59	4.98

Rat No.	Sex.	Age.	Weight.	Surface.	Hair.	Hair.	
						Per gm. body weight.	Per sq. cm. body surface.
		<i>wks.</i>	<i>gm.</i>	<i>sq. cm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>
22	F.	15	107.7	257.1	0.5827	5.41	2.26
23	"	15	80.0	210.9	0.4022	5.02	1.90
25	"	15	105.6	253.8	0.7735	7.32	3.04
27	"	15	112.0	263.9	0.6684	5.96	2.53
31	"	21	150.7	321.7	1.3335	8.84	4.14
34	"	21	119.0	274.8	0.5364	4.50	1.95
35	"	21	160.6	335.6	0.7522	4.68	2.24
24	M.	15	127.4	287.6	0.5920	4.64	2.05
26	"	15	122.4	280.0	0.7581	6.19	2.70
28	"	15	110.0	260.8	0.5927	5.38	2.27
29	"	15	117.3	272.2	0.7420	6.32	2.72
30	"	15	141.9	309.0	0.9797	6.90	3.17
32	"	21	156.4	329.7	0.5005	3.20	1.51
33	"	21	133.5	296.7	0.7345	5.50	2.47
Average.....						5.70	2.49

average weights of only 124.7 (males) and 101.4 (females) gm. in a period of 11 weeks on the experimental diet, while as the amount of casein supplementing the basal diet was increased to 2, 8, and 16 per cent, the final body weights were 161.4, 249.9, and 270.5 gm. respectively for the males and 147.9, 154.7, and 183.5 gm. for the females. A comparison of the weights of Groups I and VII will show that the animals of the latter group were able to

TABLE VI.
Group IV, Diet E. Adequate Protein and Cystine Content.

Rat No.	Sex.	Age.	Weight.	Surface.	Hair.	Hair.	
						Per gm. body weight.	Per sq. cm. body surface.
		<i>wks.</i>	<i>gm.</i>	<i>sq. cm.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>
64	F.	15	188.0	372.8	1.8240	9.70	4.89
68	"	15	175.5	356.1	2.1815	12.43	6.12
69	"	15	177.2	358.3	2.0180	11.38	5.63
70	"	15	193.5	380.0	2.2212	11.47	5.84
74	"	21	176.2	357.0	1.9235	10.91	5.38
75	"	21	179.2	361.0	2.2270	12.42	6.16
76	"	21	169.1	347.3	2.0487	12.11	5.89
77	"	21	187.8	372.5	2.8315	15.07	7.60
65	M.	15	213.0	404.3	2.5055	11.76	6.18
66	"	15	252.6	453.9	3.6814	14.57	8.11
67	"	15	241.6	430.6	2.9764	12.31	6.75
71	"	15	336.6	549.7	4.7230	14.03	8.59
72	"	15	309.0	519.2	3.9308	12.72	7.57
73	"	21	297.7	506.4	4.2160	14.16	8.32
Average.....						12.50	6.64

make as great increments in body weight in 2 to 3 weeks as did the rats on the inadequate low protein cystine-poor diet in the longer period of 11 weeks. It will be also observed that the animals which received the highest percentage of casein as a supplement to the basal diet (Group IV, Diet E) were able to grow practically as rapidly as the animals maintained on the stock diet throughout the experimental period.

Detailed data are given for the young rats (Table III), the stock rats killed at a body weight of 120 gm. (Table IV), and the two

TABLE VII.
Influence of Diet on Production of Hair.

	Hair from:					
	Males.		Females.		All animals.	
	Per gm. body weight.	Per sq. cm. body surface.	Per gm. body weight.	Per sq. cm. body surface.	Per gm. body weight.	Per sq. cm. body surface.
Group I, Diet B.						
Minimum.....	3.20	1.51	4.50	1.90		
Maximum.....	6.90	3.17	8.84	4.14		
Average.....	5.44	2.41	5.98	2.58	5.70	2.49
No. of rats.....	7	7	7	7	14	14
Group II, Diet C.						
Minimum.....	6.02	2.73	6.20	2.89		
Maximum.....	10.93	5.54	8.82	4.08		
Average.....	7.54	3.69	7.67	3.59	7.60	3.64
No. of rats.....	7	7	7	7	14	14
Group III, Diet D.						
Minimum.....	11.73	6.39	9.10	4.23		
Maximum.....	16.78	9.55	12.15	6.10		
Average.....	13.58	7.61	10.92	5.24	11.49	5.77
No. of rats.....	3	3	11	11	14	14
Group IV, Diet E.						
Minimum.....	11.76	6.18	9.70	4.89		
Maximum.....	14.57	8.59	15.07	7.60		
Average.....	13.25	7.58	11.93	5.93	12.50	6.64
No. of rats.....	6	6	8	8	14	14
Group V, 30 days old.						
Minimum.....	4.75	1.57	5.23	1.81		
Maximum.....	9.99	3.26	8.33	3.03		
Average.....	7.05	2.39	6.67	2.33	6.90	2.37
No. of rats.....	6	6	4	4	10	10

TABLE VII—*Concluded.*

	Hair from:					
	Males.		Females.		All animals.	
	Per gm. body weight.	Per sq. cm. body surface.	Per gm. body weight.	Per sq. cm. body surface.	Per gm. body weight.	Per sq. cm. body surface.
Group VI, stock diet.						
Minimum.....	14.21	7.90	9.59	4.71		
Maximum.....	21.08	13.11	14.30	7.05		
Average.....	16.40	9.71	12.51	6.12	14.18	7.66
No. of rats.....	6	6	8	8	14	14
Group VII, stock diet; 6 to 7 wks. old.						
Minimum.....	10.10	4.41	10.50	4.45		
Maximum.....	12.69	5.59	14.74	6.39		
Average.....	11.80	5.16	11.48	4.90	11.59	4.98
No. of rats.....	3	3	6	6	9	9
Group VIII, adult females.						
Minimum.....			8.04	4.47		
Maximum.....			12.07	6.42		
Average.....			10.28	5.51		
No. of rats.....			7	7		

experimental groups which received the least and greatest percentage of casein as a supplement to the basal diet (Tables V and VI). These groups we consider the most significant in the interpretation of our results. Since the animals which received diets containing intermediate amounts of casein and the two remaining control groups showed similar variations, it is considered sufficient to include these groups in the table in which the values for all the experimental animals are summarized (Table VII). The variations between the individual animals of the groups presented in the detailed tables are typical of the degree of variation observed in all the experiments.

As detailed in Table III, young white rats, killed at the age at which our feeding experiments with the other groups were begun,

had produced an amount of hair equivalent to 6.89 mg. per gm. of body weight or to 2.37 mg. per sq. cm. of body surface. These figures may be regarded as typical of the first or puppy coat of hair developed. According to Greenman and Duhring (15), this coat is succeeded by the adult type of hair which is heavier and slightly yellow tinted, shortly after the age of 6 weeks. This change in the character of the hair at the age of 6 or 7 weeks is shown in Table IV, in which the results with rats maintained on the stock diet until they have reached a weight of 120 gm. (age 6 to 7 weeks) are presented. These rats which are in the period of the active production of the adult coat of hair have elaborated a coat of hair whose weight in proportion to the body weight or body surface is *nearly double* that of the young rats of Group V (Table III). That the character of this coat is similar to that of older animals is shown by a comparison with the data for Group VI (Table VII). This latter group was fed the stock diet until the animals approached maturity and had passed through the period of manufacture of an adult coat. The amount of hair in proportion to body weight or surface was somewhat greater than with the animals of Group VII, but the difference was not as marked as the difference between Groups V and VII. It will also be noted that animals which received in the diet amounts of protein sufficient to produce normal or nearly normal rates of growth have also produced similar coats of hair, the heavier adult type (Group IV, Table VI; Groups III and IV, Table VII). From the above considerations, there would appear to exist two types of coats, a light puppy coat produced first in youth and a second heavier coat, which begins to be formed normally at about the 6th or 7th week of life and which in relation to the body weight or surface of the animal remains nearly the same throughout the later period of life, at least as far as our measurements demonstrate.

The animals of Group I (Table V), which received a diet inadequate in its protein (and cystine) content and which were able during a period of 11 weeks to make approximately the same gain in weight as the control animals (Group VII) on a stock diet in 2 to 3 weeks, did not produce a coat of hair of the *type grown by normal animals of either the same weight (Group VII) or age (Group VI)*. The average weight of the hair was 5.7 mg. per gm. of body weight, a figure more nearly comparable to the corre-

sponding weight of hair of the young rats at the age of 30 days (Group V), 6.8 mg., than to the figure 11.59 for normal rats of the same weight or 14.18 for normal rats of the same age. The rats of Group II, which also received a diet whose content of protein (and cystine) was inadequate for a normal rate of growth, although they were able to produce slightly heavier coats of hair (Table VII) than did the rats of Group I, did not show a coat of the "adult" type, such as that produced by animals of Groups III and IV (Table VII) maintained on diets satisfactory in their content of protein. It would thus appear that when the protein (and cystine) content of the diet is not adequate for both general somatic development and hair production, the demands for the production of the less essential biological structure, the hair, are not met and a coat of hair normal for an animal of that particular age or weight cannot be produced.

Further evidence in support of the theory that the demands for cystine for growth and body development take precedence over the requirement for the production of hair is afforded by the data obtained with Group VIII (Table VII), adult females¹ of 12 to 14 months of age. These animals were breeding stock which, as is usual in rat colonies, had successfully completed a series of cycles of pregnancy and lactation with short time intervals between each. In these animals the demand for protein for the building of fetal tissue and for milk production must have been great. If our hypothesis is correct, in such animals opportunity for replacement of hair lost through shedding should have been limited owing to the demands for cystine for the other purposes mentioned. In these groups the amount of hair was somewhat less than that present in the pelts of any other adult groups except those groups (Nos. I and II) in which the dietary supply of protein (and cystine) was known to be inadequate.

It is, of course, impossible to determine the amount of the hair shed by the animals during the period of our experiments. The figures given represent merely the weight of hair present in the pelt at the time the animals were sacrificed. It was noted that all the experimental groups shed hair during the period of study

¹ No sexual difference in the rate of the growth of hair was evident in the series of experimental animals. The data for the animals are summarized according to sex, however, in Table VII.

except Groups I and II on diets inadequate in protein content. These animals lost little hair and were apparently able to add but little to the total hair present when the experiment was started.

Further studies have indicated that not only the amount of hair produced but also the chemical composition of the hair was influenced by the protein (and cystine) content of the diet. These results will be presented in a later publication.

SUMMARY.

1. The production of the cystine-rich epidermal tissue, the hair, has been studied in young white rats fed diets in which the low cystine content of the protein fed (casein) was the chief factor limiting the rate of growth.

2. The amount of hair produced, like the general somatic development, was related to the protein (and cystine) content of the diet, but under the experimental conditions of the present study, the demands for protein (and cystine) for the growth of the hair appeared to be secondary in importance to the demands for growth of the body with its more essential tissues.

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CHEMICAL STUDIES OF MUSCLE CONTRACTURE.

III. THE CHANGE IN GLYCOGEN DURING SHORTENING PRODUCED BY TETANUS TOXIN.

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(Received for publication, March 21, 1929.)

In a preceding paper (2) negative findings were reported for any change in the lactic acid content of mammalian muscle during contracture produced by tetanus toxin. The study has been extended to glycogen and although definite changes were found, a simple interpretation of the changes could not be made on the basis of muscle shortening.

Analyses made by Ishizaka (which were reported by Fröhlich and Meyer (4)) showed that the glycogen content of gastrocnemii of cats was higher in muscles shortened through the agency of tetanus toxin than in untreated control muscles from the opposite side of the animal. The maximum amount found in the tetanus muscles was 0.37 per cent. Amounts in the control muscles were lower and in one instance only 0.01 per cent was found. These values are lower than those usually found in normal animals and suggest that some change in glycogen metabolism takes place during poisoning with tetanus toxin.

Although the mechanism of the formation of a contracture by tetanus toxin has not been satisfactorily explained, Ranson and Morris (10) and Ranson and Sams (11) have shown that the phenomenon can be divided into two stages. During the first stage, the muscle is kept in a shortened state by nerve impulses which reach it through the motor nerve. During the second stage the contracture persists after profound general anesthesia or section of the motor nerve and is no longer dependent upon nerve impulses.

Experiments by Wertheimer (13), Hoffman and Wertheimer (6),

and Embden and Habs (3) lead to the conclusion that the glycogen metabolism of striped muscle is intimately dependent upon its motor innervation. In Wertheimer's experiments, animals were fasted and given phlorhizin after one sciatic nerve was cut, and even though the glycogen in the innervated side fell to a low level, the amount found in the denervated muscles was comparable to that found in normal animals. Both Hoffman and Wertheimer and Embden and Habs found that the amount of glycogen in muscle could be increased by faradic treatment.

If in the establishment of a tetanus contracture, motor impulses are present to an extent greater than normal, one might expect a reduction in glycogen on the affected side. This result (opposite from Ishizaka's findings) was reported by Wertheimer in 1928 (14), although a reduction was not invariably present. Cutting the sciatic nerve of the uninjected limb did not cause a change in results. This last report appeared during the time our work was in progress so we have abridged our review of the literature and refer the reader to Wertheimer's article for a more complete discussion of the theories involved.

A complete review of the subject of local tetanus will be found in the article listed under reference (9).

EXPERIMENTAL.

Guinea pigs, white rats, and rabbits were used for experiments with tetanus toxin, and cats, for three experiments in which contractures were produced by cutting the dorsal roots of spinal nerves.

The toxin^{1,2} was injected aseptically into the popliteal space of one limb, and after the contracture had developed, both gastrocnemii were removed and prepared for glycogen determinations. The soleus muscle was usually removed with the gastrocnemius. Muscles were frozen in the same manner as for lactic acid determination (1). After removal from the animal they were cut while frozen into 0.1 to 0.2 mm. cross sections and the slices put into ice-cold 30 per cent sodium hydroxide solution. About 5 cc.

¹ For tables of relative susceptibilities of animals see Rosenau, M. J., and Anderson, I. F., *Bull. Hyg. Lab., U. S. P. H. S.*, **43**, 25 (1908).

² The tetanus toxin for this work was furnished by Parke, Davis and Company.

of the strong alkali solution were used for each gm. of muscle. Weights were obtained by weighing the flask before and after it received the sample.

The analyses for glycogen were carried out according to the principles established by Pflüger, and sugar was determined after acid hydrolysis by Somogyi's (12) modification of the Shaffer-Hartmann procedure.

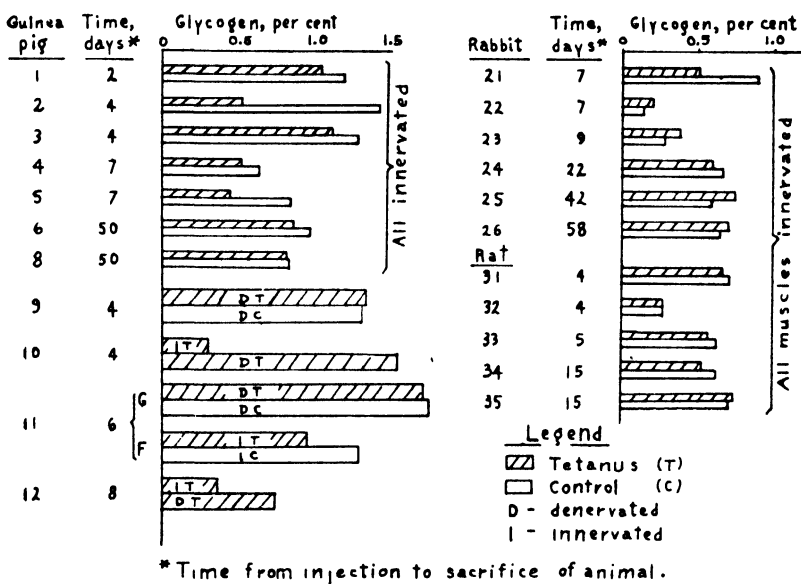


FIG. 1. Glycogen content of gastrocnemii during tetanus contracture.

Since amytal was used for the anesthetic, it seems appropriate to review the status of our knowledge concerning its effect on the metabolism of muscle glycogen. Long (7) found that general anesthesia (ether or amytal) caused a decrease in glycogen. Hinsey and Davenport (5) were not able to confirm this observation but found (unpublished data) in agreement with Long, that if a muscle is stimulated during amytal anesthesia and the glycogen thereby reduced, the original resting glycogen level is not restored for a period of 2 to 3 hours. It would seem that amytal would not affect the findings in tetanus, for the glycogen would be

neither reduced from a resting value nor increased from a decreased amount.

The cats included in the series had received no tetanus toxin but showed well defined contractures which did not relax under anesthesia. The contractures were a sequel to the cutting (intradurally) of the dorsal roots of the spinal nerves from the third lumbar to the third sacral segments. In order to favor the development of contracture a small nick was made in the dorsal portion of the posterior funiculus of the cord at the level of the third lumbar segment and on the same side as the divided roots. A description of contractures following operative procedures of similar type has been reported (8).

Fig. 1 shows that there was a wide variation in the difference in glycogen content between the two sides. The guinea pigs and rabbits exhibited the largest variations while the rats showed very little. The maximum variation (with intact innervation) occurred in Guinea Pig 2 where the glycogen on the tetanus side was only 37 per cent of that on the uninjected side. Rabbits 22 and 23 showed a reversal of the usual order in that there was less glycogen in the flaccid muscles than in those in contracture. These two animals had refused to eat for several days and the glycogen content of both sides was below normal. Perhaps the glycogen was exhausted, during the general depletion of carbohydrate stores, more rapidly from the legs used in moving about than from those which were too stiff from tetanus to be exercised. This phenomenon appears to be possible in light of Wertheimer's (13) findings that a denervated (therefore unused) muscle retains its glycogen during fasting. Rat 32 showed low glycogen also but it was equal on the two sides, hence the above explanation may not be adequate.

If the reduction in glycogen had been uniformly present in all species and in both tetanus contracture and the contracture following dorsal root section with spinal cord injury, one would be led to the conclusion that it was directly related to the contracture. Wertheimer (14) expressed the belief that the variableness and relatively small amount of reduction in glycogen indicated that the loss of glycogen had very little to do with the contracture. This irrelevancy seemed even more likely since the contractures of longer duration showed less reduction and were at the same

time associated with some atrophy of the muscle. Our results are in agreement with his conclusions, and the interpretation is further complicated by the findings in Rabbits 25 and 26. These two animals were kept for 42 and 58 days respectively and even though function of the tetanus limbs was not regained, their gastrocnemii contained more glycogen than the functioning gastrocnemii on the opposite sides.

Guinea Pigs 9 and 11 had both sciatic nerves cut at the same time that toxin was injected into only one limb. These animals show that motor innervation is an essential feature of the glycogen change. The denervated tetanus muscles contained the same amount of glycogen as the controls but coincidentally developed no contractures. Guinea Pigs 10 and 12 had only one sciatic nerve cut and tetanus toxin injected into both limbs. Here the decrease in glycogen is seen only on the innervated side. Samples IT and IC of Specimen 11 F represent opposite quadriceps femoris muscles of Guinea Pig 11, and since these muscles were innervated on both sides and toxin was present in only one limb, the innervated tetanus muscle contains less glycogen than the other, while the denervated gastrocnemii (Specimen 11 G) show no change.

The negative findings in contracture produced by dorsal root section and cord injury show that contracture can occur without significant change in glycogen in the affected muscle when the functioning companion muscle is used as a basis for comparison. Values of 0.47 and 0.45; 0.49 and 0.50; and 0.60 and 0.56 per cent were obtained for functioning and contracted muscles respectively.

SUMMARY.

1. The glycogen content of guinea pig and rabbit gastrocnemii is usually reduced by tetanus toxin during the early stages of contracture.
2. In rats the glycogen shows little change under the same treatment.
3. Contractures resulting from dorsal root section of spinal nerves showed no change in glycogen.
4. The variability in reaction of the glycogen content among different species and during different stages of tetanus contracture

indicates that it does not have a cause and effect relationship to the contracture.

5. Tetanus toxin causes no change in the glycogen content of denervated muscles.

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THE PREVENTION OF THE TETANY OF PARATHYROID-ECTOMIZED DOGS.

I. COD LIVER OIL. WITH A NOTE ON THE EFFECT OF COD LIVER OIL ON CALCIUM ASSIMILATION.*

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That the parathyroid glands are intimately concerned with calcium-phosphorus metabolism and with the maintenance of a normal concentration of calcium in the blood and, presumably, in other body fluids and tissues seems definitely established. It seems equally certain that, unless the ratio of calcium to phosphorus in the diet is carefully regulated, proper calcification of the bones of young animals can be obtained only if the organism is supplied with the antirachitic vitamin, either in the food or by direct irradiation of the animal. It is, therefore, to be expected that furnishing the antirachitic vitamin, in either of these ways, to parathyroidectomized dogs should, in some manner, affect the nature of the symptoms observed. Since the excretion of calcium and phosphorus is diminished by the removal of the parathyroid glands (1-3) and increased by the administration of parathyroid extract (4-6), and since cod liver oil promotes calcification, it might be expected that the feeding of cod liver oil to parathyroidectomized dogs would intensify the symptoms. On the other hand, the therapeutic usefulness of cod liver oil in infantile tetany (7, 8) might lead one to expect a similar curative or preventive action in parathyroidectomized dogs.

It may not be amiss to point out here that, although the immediate cause of tetany, both in infants and in parathyroidectomized dogs, is probably the low concentration of calcium in the body

* A preliminary report was read before the American Society of Biological Chemists at Ann Arbor in April, 1928.

fluids, the conditions responsible for this immediate cause may be quite different. There are probably some cases of infantile tetany in which the disorder is essentially one of hypoparathyroidism but this is almost certainly not true of all.

Several years ago, we fed cod liver oil to several of our thyro-parathyroidectomized dogs. Of these, three received cod liver oil for 2 days before the operation and one for 8 days. All received bone ash. Even though large amounts of cod liver oil, 40 to 50 cc., were administered, all of the dogs developed tetany. One died in tetany on the 6th day. Another refused food after about 2 weeks of intermittent tetany and was killed on the 19th day. The serum contained 9.51 mg. of calcium and 10 mg. of inorganic phosphorus per 100 cc. A third recovered after 2 days of mild tetany and remained in good condition until the 19th day, when it was killed. The serum then obtained contained 13.7 mg. of calcium and 2.8 mg. of inorganic phosphorus per 100 cc. The fourth dog received cod liver oil for 8 days before the operation, as well as thereafter. As tetany had not appeared on the 4th day, the bone ash was replaced by infusorial earth 2 days later, in spite of the continued administration of cod liver oil, the animal was in tetany. This then disappeared for 3 days to recur later. The dog then refused food and vomited what was given by stomach tube. Although not in tetany at the time, the dog was bled to death on the 13th day. The serum contained 6.81 mg. of calcium and 5.16 mg. of inorganic phosphorus per 100 cc.

At the time, it seemed to us that the recovery of three dogs from tetany could be best explained by the assumption that we had not succeeded in removing all the parathyroid tissue and that the remaining tissue had hypertrophied sufficiently to supply enough hormone to keep the animals free from tetany. In two of these animals, the final concentration of calcium in the serum was normal—in one, indeed, distinctly above normal. In the third, it was at just about the "tetany level." This was in the animal that had received the longest course of preparatory treatment and had also received large amounts of cod liver oil after the operation. Certainly, there was in these experiments no indication of any marked preventive action by cod liver oil. They were, therefore, discontinued.

Since then, Jones (9) has reported the results of a series of

experiments on dogs in which the administration of 20 cc. of cod liver oil daily for 2 weeks before the operation sufficed to prevent the appearance of tetany after thyroparathyroidectomy. The administration of cod liver oil after the operation was not necessary, though no ill effect was observed in the one experiment in which this was done. The concentration of calcium in the serum fell, though not so rapidly as in dogs that developed tetany and finally reached very low values. The two animals that had received the cod liver oil for only 7 days before the operation showed slight tetany but only once in each case and lived for 17 and 24 days, respectively. The two dogs that received cod liver oil only after the operation developed tetany on the 2nd day and died during the following night.

Results such as these could not but be of interest to all those interested in the physiology of the parathyroid glands. They seemed unequivocal and demanded explanation. We determined to study the metabolic changes involved.

Methods.

The general conduct of the experiments and the methods of analysis were those described in a previous publication (3).

In some of the experiments, ferric hydroxide was added to the diet in order to compare Bergeim's technique (10) with that long used in this laboratory; *viz.*, the use of infusorial earth, the determination of the silica content of the feces and of the ratio of the amounts of constituents regarding which information is desired to the amount of silica. There seemed to be no advantage in the use of Bergeim's modification. It has, theoretically, a slight disadvantage in that a certain amount of iron is dissolved, absorbed, and reexcreted. The infusorial earth remains undissolved. The only disadvantage is that there is a slightly greater error in the determination of silica than in that of iron ¹

In order to secure diets that the dogs would eat, it was generally necessary to include some meat. The amount was usually small, 3 to 7 gm. per kilo of body weight, and seemed to have little effect on the incidence of tetany. In fact, the dogs that received

¹ Very recently, silica, in the form of washed sea sand, has been found to be as satisfactory as iron hydroxide in the determination of the digestibility of protein (Gallup, W. D., *J. Biol. Chem.*, **81**, 321 (1929)).

more meat, 13 to 50 gm. per kilo, were among those that showed the least marked tetany.

DISCUSSION.

Much to our surprise, we found that even large doses of cod liver oil exercised no marked protective action. Several of the dogs lived a rather long time but they all showed tetany at one time or another. In only two of the nine experiments of the first series was tetany delayed until appreciably later than is usual in our experience. In a tenth experiment (Dog 85), maize oil was substituted for the cod liver oil beginning with the day of the operation. Severe tetany occurred on the 2nd day, and 24 hours later the dog was dead.

Since we had rather longer survivals in our earlier experiments, already referred to, in which bone ash was added to the diet, we performed three additional experiments in which bone ash was used. Two of these dogs developed tetany but later recovered. One of these two (Dog 80) received calcium lactate, which probably aided in the recovery. The third dog (No. 84) did not develop tetany but was so sick at the time of the operation and thereafter that we question the significance of the experiment.

These results seemed to be in almost complete contradiction to those reported by Jones. It was only as a result of a study of the changes in the calcium and phosphorus excretion and of the concentration of these substances in the serum that we were enabled to arrive at an interpretation that seems to reconcile the two sets of experimental results as well as the irregularities in our own. This interpretation seems consistent with the work of others on the protective action of cod liver oil, its effect on the calcium and phosphorus metabolism of normal animals, etc. We will first discuss our own experiments.

A summary of these, showing the amount of cod liver oil fed, the length of the fore period, the number of days before the appearance of tetany, analyses of the serum at different times, etc., is presented in Table I. Tables II to IV, each summarize the results of metabolic studies on one dog. In order to economize space, only the averages for the fore periods are presented. In Table V, we have brought together the results of the determinations in those experiments in which the feces were not analyzed. The

data presented in these tables seem to indicate the mechanism by which some of the animals were enabled to survive for a considerable period.

For instance, in Table II there are presented the results of the analyses of the urine and feces of Dog 57. This animal remained free from tetany for 6 days after the operation. The diet was then changed to 300 gm. of beef heart and 25 gm. of infusorial earth. Tetany appeared 4 days later. It was relieved by the administration of calcium lactate and did not recur when this was discontinued. However, sneezing, a frequent sign of parathyroid insufficiency, was very marked.² The reason for the delay in the appearance of tetany was not immediately apparent. However, when we came to analyze the feces, we were struck by the large amounts of calcium that they contained. Investigation showed that the infusorial earth used in this experiment contained large amounts of calcium, apparently as calcium carbonate.⁴ In this manner, the dog received approximately 1 gm. of calcium per day, so that the ratio of calcium to phosphorus in the diet was about 6:1. The analyses of the urine showed the usual sharp drop in the excretion of phosphorus and the analyses of the feces indicated a retention of about 0.1 gm. of calcium per day. This was more than sufficient to combine with all the retained phosphorus. Later, when the animal was placed on a meat diet and the phosphorus content of the food greatly increased thereby, the calcium in the infusorial earth seemed to become less available, the concentration of diffusible calcium in the tissues was no longer maintained, and tetany appeared. This was relieved by the administration of calcium lactate.

The next dog, No. 58, (Tables I and III) showed the first signs

² At this point we may digress for a moment to point out that sneezing and wheezing, very similar to those observed in clinical asthma are of frequent occurrence in latent tetany. The use of calcium lactate in those cases of asthma in which a definite allergic cause cannot be demonstrated is worthy of consideration. The previous negative results with the use of calcium salts in asthma may have been due to insufficient dosage or to unsuitable administration (Pottenger (11), Kayser (12), Roe and Kahn (13)).

⁴ This was the only batch of infusorial earth that contained considerable quantities of calcium and it was used in only this one experiment of this series.

TABLE I.
Summary of Experiments with Thyroparathyroidectomized Dogs on Diets Containing Cod Liver Oil.

Dog No.	Cod liver oil.	Days on diet before operation.	Days to appearance of tetany.	Analysis of serum.								Remarks.
				At operation.				Subsequent.				
				Protein.	Ca	In-organic P.	Days after operation.	Protein.	Ca	In-organic P.		
57	5.0	12	10				(16)	7.28	4.80		Tetany relieved by Ca lactate. Recurred. Again relieved.	
58	5.1	15	4		10.3		5	5.74	4.86		"	
62	4.0	18	13				(7)	6.28	5.21	4.97	Cod liver oil discontinued 7 days after operation. Tetany on 13th and 14th days.	
							14	6.22	4.86	4.86	Severe tetany.	
64	2.8	16	3	8.11	13.7		3	6.29	4.64	7.69	Severe tetany.	
65	2.5	18	5	5.66	12.2	6.28	(3)	5.73	8.25	8.25	Severe tetany.	
							5	6.30	6.33	8.06	Severe tetany.	
66	2.7	27	4 (?)	6.12	11.7	5.80	(3)	6.84	6.70	11.0	Dead on 4th day. Position indicated tetany.	
											Severe tetany.	
67	3.0	17	2	7.00	11.2	3.53	2	7.61	6.25	1.42	No tetany on 3rd day, but condition of cage indicated tetany previous night. Severe on 4th day.	
68	3.2	30	2 (?)	5.90	11.5	3.93	(3)	6.43	6.97	8.54	Severe tetany.	
							4	5.51	5.26	13.4	Severe tetany.	
70	5.9	18	2	5.52	9.8	2.49	2	4.77	4.47	2.99	Severe tetany.	

85	3.0	18	2	4.48	10.0	5.11†	2	4.46	4.98	8.12	Severe tetany. Short attacks of tetany 2nd to 5th days. Then refused ration. Fed meat and Ca lactate 7th to 13th days; then meat only. Slight tetany 7th to 11th days. Sick at time of operation.
80	2.7	17	2	4.94‡	11.1†	4.00‡	(3)	5.67	7.14	5.78	
							(5)	5.46	6.62	5.56	
				5.59	10.5	4.35	(14)	5.58	6.97	5.52	
83	3.0	23	7	5.87	10.9	3.26†	(21)	5.07	6.18	6.45	
							(3)	5.51	6.78	4.79	
							(21)	5.86	6.53	4.07	
84	3.0	29		4.76	9.1	5.22	(2)	4.71	4.39	8.18	

* Figures in parentheses indicate that tetany was not present at the time the blood was drawn.

† Maize oil was substituted for cod liver oil after the operation.

‡ The blood was drawn after 6 days of cod liver oil feeding. 7 of the 20 cc. of cod liver oil were then replaced by 7 cc. of cottonseed oil containing 7 mg. of irradiated ergosterol.

TABLE II.

Dog 57.—Female, weight 12 kilos. Diet, 60 cc. of cod liver oil, 60 gm. of cracker meal, 10 gm. of dried meat residue, 25 gm. of infusorial earth, 3 gm. of $\text{Fe}(\text{OH})_3$, since January 12, 1927. Thyroparathyroidectomy, January 24.

Date.	Urine.			Feces.							
	N	P	Ca	SiO ₂	Fe	P	Ca	Per gm. SiO ₂ .		Per gm. Fe.	
								P	Ca	P	Ca
1927	gm.	gm.	gm.	gm.	gm.	gm.	gm.	mg.	mg.	mg.	mg.
Average, Jan. 19– 23.	2.46	0.0337	0.0091	19.1	1.77	0.123	0.99	6.4	52	70	556
Jan. 24	2.23	0.0041	0.0120	11.4	1.25	0.085	0.53	7.5	46	69	425
" 25	4.26	0.0074	0.0130	14.8	1.42	0.093	0.72	6.3	49	65	511
" 26	2.29	0.0040	0.0004	17.8	1.89	0.145	0.86	8.1	49	76	453
" 27	2.56	0.0049	0.0005	22.2	2.18	0.155	1.26	7.0	57	71	576
" 28	1.66	0.0024	0.0004	15.4	1.26	0.088	0.49	5.7	32	70	392
" 29	2.18	0.0034	0.0002	14.6	1.00	0.080	0.57	5.5	39	80	570

TABLE III.

Dog 58.—Female, weight 16 kilos. Diet, 82 cc. of cod liver oil, 126 gm. of cracker meal, 35 gm. of infusorial earth, since December 18, 1926. Thyroparathyroidectomy, January 3, 1927. Slight tremor, January 6; tetany, January 7.

Date.	Urine.			Feces.						
	N	P	Ca	SiO ₂	P	Ca	Per gm. SiO ₂ .			
							P	Ca		
1927	gm.	gm.	gm.	gm.	gm.	gm.	mg.	mg.		
Average, Dec. 28– Jan. 2.	2.36	0.134	0.0013	27.2	0.096	0.204	3.5	7.5		
Jan. 3	2.65	0.014	0.0013	14.7	0.066	0.144	4.5	9.8		
" 4	2.58	0.007	0.0008	16.9	0.076	0.135	4.5	8.0		
" 5	2.75	0.007	0.0006	33.9	0.150	0.224	4.4	6.6		
" 6	2.68	0.007	0.0002	33.2	0.149	0.230	4.5	7.0		
" 7	1.55	0.004	0.0006	23.6	0.102	0.158	4.3	6.7		

of tetany on the 4th day after the operation. Quite severe tetany was observed on the next day. The urine showed the usual fall in the amount of phosphorus excreted. This was accompanied by a small increase in the excretion of phosphorus in the feces and by a slight, though distinct, retention of calcium.

In the next experiment, upon Dog 62, (Tables I and IV) analyses were unfortunately not begun until only 3 days before the operation. However, it appears from an inspection of Table IV that there was a decrease in the excretion of phosphorus and of calcium

TABLE IV.

Dog 62.—Male, weight 7.6 kilos. Diet, 30 cc. of cod liver oil, 380 gm. of beef heart, 10 gm. of infusorial earth, since May 19, 1927. Thyroparathyroidectomy, June 8; doubtful tremor, June 13

Date.	Urine			Feces				
	N	P	Ca	SiO ₂	P	Ca	Per gm. SiO ₂	
							P	Ca
1927	gm	gm.	gm	gm	gm	gm	mJ	mJ
Average, June 5– 7.	8.25	0.633	0.0006	7.17	0.069	0.110	9.6	15.3
June 8	9.69	0.426	0.0009	11.47	0.116	0.153	9.9	13.3
" 9	7.53	0.455	0.0006					
" 10	8.73	0.580	0.0002					
" 11	6.78	0.469	0.0004					
" 12	11.53	0.761	0.0003					
" 13	7.83	0.510	0.0003					
" 14	8.47	0.526	0.0004	5.75	0.058	0.078	10.2	13.5

in the feces. It is even more evident that the diminution in the excretion of phosphorus in the urine was much smaller than is usual in parathyroidectomized dogs. It would seem that not all the parathyroid tissue had been removed and that what remained was, for a week, sufficient to prevent a too rapid fall in the concentration of calcium in the plasma. Possibly the continued administration of cod liver oil helped to attain this result, for, when it was discontinued, the concentration of calcium fell somewhat and tetany developed.

TABLE V.

*Excretion of Nitrogen and Inorganic Phosphorus in Urine of Parathyroidectomized Dogs Receiving Cod Liver Oil.**

Dog No.....	64		65		66		68	
Fore period, days.....	4		6		6		6	
	N	Inor- ganic P.	N	Inor- ganic P.	N	Inor- ganic P.	N	Inor- ganic P.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Average for fore period.....	5.26	0.178	8.03	0.221	8.53	0.225	6.25	0.169
Operation.....	9.16	0.167	8.19	0.00†	7.08	0.00†	7.30	0.00†
Days following operation:								
1.....	8.62	0.216	8.71	0.023	7.99	0.00†	7.08	0.00†
2.....	Severe tetany.		5.55	0.048	8.10	0.00†	Severe tetany.	
3.....			Lost.		Found dead.			
4.....			Severe tetany.					
Dog No.....	67		70		85		80	
Fore period, days.....	6		7		3		2	
	N	Inor- ganic P.	N	Inor- ganic P.	N	Inor- ganic P.	N	Inor- ganic P.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Average for fore period.....	15.6	0.437	2.11	0.191	1.98	0.245	4.98	0.163
Operation.....	7.5	0.071	1.59	0.017	2.72	0.069	5.38	0.081
Days following operation:								
1.....	Severe tetany.		3.86	0.009	2.16	0.118	4.25	0.019
2.....			Severe tetany.		Severe tetany.		4.75	0.039
3.....							3.81	0.066
4.....							5.51	0.114
5.....							5.16	0.120
6.....							Some tetany, every day, 2nd to 6th days.	

* For experiments in which the excretion of calcium was also determined see Tables II to IV.

† The amounts were too small to be determined by Briggs' method.

TABLE V—*Concluded.*

Dog No.....	83		84	
Fore period, days.....	4		5	
	N	Inorganic P.	N	Inorganic P.
	gm.	gm.	gm.	gm.
Average for fore period.....	3.96	0.262	3.96	0.263
Operation.....	6.95	0.247	2.80	0.027
Days following operation:				
1.....	6.78	0.305	4.62	0.136
2.....	7.09	0.299	Did not develop tetany, but was very sick. See text, p. 517.	
3.....	6.77	0.246		
4.....	8.74	0.377		
5.....	10.99	0.682		
6.....	Some twitching on 2nd day, short attack of tetany on 3rd day.			

The diets fed the dogs are as follows:

Dog 64.—20 cc. of cod liver oil, 50 gm. of beef heart, 40 gm. of dried meat residue, 30 gm. of cracker meal, 10 gm. of infusorial earth.

Dog 65.—25 cc. of cod liver oil, 50 gm. of beef heart, 60 gm. of dried meat residue, 30 gm. of cracker meal, 10 gm. of infusorial earth.

Dog 66.—30 cc. of cod liver oil, 50 gm. of beef heart, 30 gm. of cracker meal, 60 gm. of dried meat residue, 18 gm. of infusorial earth.

Dog 67.—60 cc. of cod liver oil, 100 gm. of beef heart, 110 gm. of dried meat residue, 50 gm. of cracker meal, 25 gm. of infusorial earth.

Dog 68.—25 cc. of cod liver oil, 50 gm. of dried meat residue, 25 gm. of cracker meal, 13 gm. of infusorial earth.

Dog 70.—65 cc. of cod liver oil, 40 gm. of beef heart, 50 gm. of Shredded Wheat, 15 gm. of infusorial earth.

Dog 80.—13 cc. of cod liver oil, 7 mg. of irradiated ergosterol, 15 gm. of beef heart, 30 gm. of dried meat residue, 30 gm. of cracker meal, 3 gm. of dried yeast, 2 gm. of salt mixture,* 10 gm. of bone ash.

Dog 83.—31 cc. of cod liver oil,† 155 gm. of beef heart, 42 gm. of Shredded Wheat, 10 gm. of bone ash.

Dog 84.—20 cc. of cod liver oil, 125 gm. of beef heart, 30 gm. of Shredded Wheat, 8 gm. of bone ash.

Dog 85.—25 cc. of cod liver oil, 25 gm. of beef heart, 70 gm. of Shredded Wheat, 2.5 gm. of salt mixture, 15 gm. of infusorial earth.

* The salt mixture was that described by Cowgill (14).

† Replaced after the operation by 31 cc. of maize oil.

In Table V we have brought together the results of the analyses in those experiments in which the feces were not analyzed and in which only the nitrogen and the inorganic phosphorus of the urine were determined. Dogs 64, 65, 67, 68, 70, and 85 all showed marked decreases in the excretion of inorganic phosphorus in the urine after the operation and developed tetany quite promptly.

We now come to the three dogs that received bone ash (Table V). The first of these, No. 80, received 20 cc. of cod liver oil per day for 18 days and then 13 cc. of cod liver oil and 7 mg. of irradiated ergosterol in 7 cc. of cottonseed oil. The operation, performed on June 11, was followed by the usual fall in the excretion of phosphorus in the urine. Slight tetany was observed on the evening of June 13 and severe tetany on June 14 and 15. Another attack on June 16 was followed by recovery but the animal refused food on June 17. When food was given by stomach tube on June 18, it was vomited. After that, the dog received 20 gm. of calcium lactate and 200 gm. of beef heart daily for 6 days. On June 25, 24 hours after the last feeding, a sample of blood was drawn. Analysis of the serum showed it to contain 5.58 per cent of protein, 6.97 mg. per cent of calcium, and 5.52 mg. per cent of inorganic phosphorus. The calcium lactate was then discontinued. On July 2, the serum contained 5.07 per cent of protein, 6.18 mg. per cent of calcium, and 6.45 mg. per cent of inorganic phosphorus. The dog was killed on July 5, without showing any other signs of tetany.

In another dog, No. 83, the use of cod liver oil was discontinued after the operation. Maize oil was fed in its stead. The effect of the parathyroidectomy on the phosphorus excretion was masked by the increased metabolism. This was probably due to the large hematoma that developed at the site of the operation. There was, however, a decrease in the ratio of inorganic phosphorus to nitrogen in the urine. There was some twitching on the 2nd day and a short attack of tetany on the 3rd day after the operation. There was no tetany during the next 2 days. The bone ash was then replaced by infusorial earth. On the 2nd day thereafter, the 7th after the operation, tetany reappeared. The use of cod liver oil was again resumed but the tetany continued. On the 10th day after the operation, the infusorial earth was replaced by bone ash. By the next day, the dog's condition had

improved and on the day thereafter tetany had practically disappeared. On the following day, the bone ash was replaced by infusorial earth. 2 days later there was some transitory twitching, but thereafter there was no sign of tetany although the cod liver oil was replaced by maize oil on the 17th day after the operation and continued for 4 days. On the 21st day the dog was bled to death, under cocaine anesthesia. *There was no sign of tetany during the bleeding, although the serum calcium was only 6.53 mg. per 100 cc.*

Finally, we have the case of Dog 84. The urine from this animal showed a marked decrease in the excretion of phosphorus after the operation and we would, ordinarily, have expected the rapid development of tetany. But, as has already been mentioned, this dog was not in good condition at the time of the operation and it was used only because it was necessary to terminate this work at this time. The animal was very sick after the operation and could not walk. It is believed that the failure to develop tetany was due to this entirely adventitious disorder, probably due to infection. The failure to develop tetany was in our opinion, comparable to its failure to appear in jaundiced dogs (15) or to the relief from tetany afforded by the administration of morphine (16).

However, we have the distinct impression, both from these experiments and those of 5 years ago, that the combination of cod liver oil and bone ash, or other source of calcium, had a slight protective action, whereas cod liver oil alone had little or none. This may have been due to an increased store of available calcium (not necessarily circulating) at the time of the operation or to an increased availability of the calcium in the bone ash fed after the operation or to a combination of both of these or to other effects, the nature of which is not immediately apparent.

While this work was in progress, Brougher (17) published a report of a series of experiments upon dogs, of which eighteen received cod liver oil and sixteen were controls. All received about 400 cc. of milk per day after the operation. If the experimental animals refused food, they received the milk and 20 cc. of cod liver oil by stomach tube. Details were not given but it was stated that the average length of the period between thyro-parathyroidectomy and the appearance of tetany was 2.5 days

in the control animals and 8.2 days in those receiving cod liver oil. In the control animals, the concentration of calcium in the serum fell to the tetanic level in 2.5 days; in the experimental animals, in 4 days. After 30 or 40 days of continued cod liver oil administration, the serum calcium rose to practically the normal level and no tetany occurred after the cod liver oil was discontinued, except when estrual cycle, pregnancy, or infection precipitated it.

We have also been informed of the experiments of Wade (18).⁴ In these, seven dogs received 20 cc. or more of cod liver oil for at least 2 weeks before thyroparathyroidectomy and five animals were used as controls. Five of the dogs fed cod liver oil developed tetany and the possibility of its occurrence in the other two was not excluded. The experimental animals survived for from 9 to 142 days, an average of 42.3 days. In the five animals that are known to have developed tetany, this was first noticed in from 8 to 108 days, an average of 35.6 days, after the operation. This is certainly a long time and would ordinarily be taken to indicate a very high degree of protection as a result of the cod liver oil treatment. But when we find that the controls survived the operation for from 3 to 35 days, average 16.6 days, and that the date of the first observed tetany was from 2 to 31 days, average 10.8 days, after the operation, it is quite obvious that there was something quite unusual about Wade's experiments. It would seem that either he did not succeed in removing as much of the parathyroid tissue as do most others or that his uncontrolled stock diet was more protective than he believed it to be. Moreover, the control dogs were not given any other oil instead of the cod liver oil given to the experimental animals. Of necessity, they must have eaten more of the various other foods than did the experimental animals. If the need for this increased food intake led to an increased consumption of foods possessing no element of protective action—meat, for instance—the lesser resistance of the controls to the effects of the operation can easily be understood.⁵

⁴ We are greatly indebted to Dr. Preston H. Wade for his kind permission to discuss his results in advance of publication.

⁵ The same criticism of a failure to control the experiments with cod liver oil by means of others with some other oil or fat applies much more strongly to the work of Brougher and also, though to a lesser extent, to that of Jones.

There is, therefore, little in the experiments of Wade that indicates any specific protective action on the part of cod liver oil.

Quite recently, Brougher (19) has described some experiments with acterol, a preparation of irradiated ergosterol. This, in amounts equivalent in antirachitic potency to 40 cc. of cod liver oil, was administered, intravenously or subcutaneously, only *after* the operation to dogs receiving a diet including 400 to 500 cc. of milk per day. Brougher concluded that acterol prevented tetany in those animals that were able to retain the milk. This was true of six out of the ten animals used in the experiment. After from 15 to 30 days, the administration was discontinued. Two dogs then developed distemper and pneumonia, could not retain milk, and developed tetany before dying of pneumonia. The other four made a complete recovery. In all six animals the concentration of calcium in the serum returned to the normal after from 20 to 40 days of acterol administration. In a still more recent publication (20), Brougher claims that the administration of 1 ounce of cod liver oil or of 0.4 cc. of acterol (the equivalent of 40 cc. of cod liver oil) relieved tetany and shortened the coagulation time of the blood, presumably by increasing the concentration of calcium in the plasma.

Brougher does not seem to realize that, since, as he admits, acterol was of value only in animals that were able to retain milk, the recovery might have been due as much to the milk as to the acterol. He makes no statement as to controls in this paper but in a previous one (17), he mentions the use of sixteen controls on a similar diet. The period of survival is not given. It is stated that tetany appeared at an average of 2.5 days after the operation and that "Serum calcium in the controls never reached the preoperative level." In a personal communication, Brougher states that three control dogs recovered and that the other thirteen lived for an average of 25 days. Calcium lactate was administered intravenously whenever the dogs were unable to retain their food. Overdosage may have occurred and the possibility of a toxic action cannot be excluded.

The observations of Dragstedt and Sudan (21) as to the lack of protection by milk, quoted by Brougher, are not of special significance in this connection. They concern only four dogs, one of which was pregnant when the experiment began and later was

lactating. In no case was milk given before the operation. The animals had been on a calcium-poor diet before the operation (22) and their calcium depots were probably depleted. Under these conditions, large amounts of calcium lactate are required to prevent tetany. Therefore, it is not surprising that 400, 500, or even 1000 cc. of milk were not sufficient to protect against tetany. If milk had been fed before the operation as well as after, the calcium depots would have been filled and the result might have been quite different. Salvesen (23) had quite good success in preventing tetany in parathyroidectomized dogs by means of a milk diet. Nevertheless, it must be recognized that Brougher's experiments do indicate a degree of protection by the combination of acterol or cod liver oil and milk that was not obtained with milk alone, under similar conditions.

Irradiated ergosterol was also employed by Urechia and Popoviciu (24). They administered it subcutaneously and by mouth to six dogs on a mixed diet. Four died in acute tetany. The other two recovered from tetany and lived at least 2 months, with subnormal concentrations of calcium in the serum.

In our experience, even large doses of irradiated ergosterol have not been successful in relieving tetany, even when the diet contained considerable quantities of calcium. The protocols of two experiments follow.

Dog 76.—Female, weight 10 kilos. Diet, 45 gm. of Shredded Wheat, 26 gm. of dry milk, 72 gm. of lactose, 1 gm. of NaCl, 3 gm. of Fe(OH)₃, since Mar. 27, 1928. Apr. 16. Thyroparathyroidectomy. Serum contained 5.50 gm. of protein, 11.8 mg. of calcium, and 6.40 mg. of inorganic phosphorus per 100 cc. Apr. 19. Tetany. Serum contained 5.03 gm. of protein, 6.40 mg. of calcium, and 7.30 mg. of inorganic phosphorus per 100 cc. Apr. 20. Tetany. Apr. 21. Tetany. Relieved by NH₄Cl. Apr. 22, 23. No tetany. Continued NH₄Cl. Apr. 23. Slight tremor. No NH₄Cl. Apr. 25. 9.30 a.m., no tremor. Serum contained 5.66 gm. of protein, 5.25 mg. of calcium, and 8.05 mg. of inorganic phosphorus per 100 cc. 10.10 a.m., usual diet plus 200 mg. of irradiated ergosterol.⁵ 12.30 p.m., tremor; more marked later in afternoon. Apr. 26. Tremor. Refused food. The usual diet plus 100 mg. of irradiated ergosterol was given by stomach tube. A little was vomited 4 hours later. Apr. 27. Tremor. Serum contained 5.49 gm. of protein, 4.38 mg. of calcium, and 7.70 mg. of inorganic phosphorus per 100 cc. All food eaten. Apr. 28. Tremor. Serum contained 5.18 gm. of protein, 4.64 mg. of calcium, and 7.70 mg. of inorganic phosphorus per 100 cc.

Dog 77.—Female, weight 11 kilos. Diet, 45 gm. of cracker meal, 36 gm. of dry milk, 72 gm. of lactose, 1 gm. of NaCl, 3 gm. of $\text{Fe}(\text{OH})_3$, since Mar. 29, 1928. Apr. 9. Thyroparathyroidectomy. Serum contained 5.63 gm. of protein, 11.1 mg. of calcium, and 5.55 mg. of inorganic phosphorus per 100 cc. Apr. 14. Tetany, relieved by NH_4Cl . Apr. 15. No tetany. Continued NH_4Cl . Apr. 16. No tetany. Serum contained 5.73 gm. of protein, 9.38 mg. of calcium, and 4.99 mg. of inorganic phosphorus per 100 cc. Continued NH_4Cl . Apr. 17–20. No NH_4Cl . No tremor. Apr. 21, 22. No NH_4Cl . Faint tremor. Apr. 23. Faint tremor, becoming more marked as preparations were made for drawing blood. Serum contained 5.88 gm. of protein, 4.91 mg. of calcium, and 8.56 mg. of inorganic phosphorus per 100 cc. Administered NH_4Cl . Apr. 24. Usual diet plus 200 mg. of irradiated ergosterol.* Slight tremor. Apr. 25. Usual diet plus 100 mg. of irradiated ergosterol. Slight tremor. Apr. 26. Usual diet plus 100 mg. of irradiated ergosterol. Slight tremor. Serum contained 6.00 gm. of protein, 5.78 mg. of calcium, and 8.45 mg. of inorganic phosphorus per 100 cc. Apr. 27. Distinct tetany. Usual diet. Apr. 28. Marked tetany. Serum contained 5.45 gm. of protein, 4.99 mg. of calcium, and 9.39 mg. of inorganic phosphorus per 100 cc.

It may be objected that the $\text{Fe}(\text{OH})_3$ might have inactivated the irradiated ergosterol. This is unlikely, both because of the large amounts we used and because in Bergeim's experiments on rats (25) the use of $\text{Fe}(\text{OH})_3$ seems not to have interfered with the potency of the cod liver oil in promoting calcium assimilation.

The experiments of Brougher, in which the concentration of calcium in the serum finally returned to the normal are comparable to those of Swingle and Wenner with strontium lactate (26) and of Wenner with magnesium lactate (27) and with ammonium chloride (28). The fact that a normal calcium content in the serum was finally attained and maintained seems to be capable of explanation only by supposing either that some other tissue took over the parathyroid function or else that accessory parathyroid tissue was present and hypertrophied. We prefer the latter hypothesis as the simpler. Moreover, accessory parathyroid tissue has been found so often as to make it very probable that it is usually present (Marine (29, 30), Farner and Klinger (31), Shapiro and Jaffe (32), Nicholas and Swingle (33)). That it is not always discovered, even when sought, is not surprising when we recall that a bit of transplanted parathyroid tissue, so small that it could scarcely be detected with the naked eye, although its

* The irradiated ergosterol was kindly supplied by Dr. Alfred F. Hess.

location was known, sufficed to keep a dog in good condition (Halsted (34)).

In fact, we are inclined to believe that only exceptionally, if ever, does one succeed in removing *all* of the parathyroid tissue. It is probable that all one usually does is to diminish the amount of such tissue so that what remains, probably only of microscopic size, is no longer sufficient to maintain the essential calcium equilibria so that tetany results. It may be that the administration of cod liver oil stimulates parathyroid tissue, so that what is not removed is, more frequently than when cod liver oil has not been used, sufficient to prevent the appearance of tetany.

There is some direct evidence that the antirachitic vitamin, or irradiation, does stimulate the parathyroids. Grant and Gates (35), observed hyperplasia of the parathyroids, with considerable increase in weight, in rabbits subjected to ultra-violet irradiation. Such rabbits possessed some degree of protection against a partial parathyroidectomy (36).

Nonidez and Goodale (37) and Higgins and Sheard (38) found that, in chicks deprived of antirachitic vitamin and of ultra-violet irradiation, there was temporary hyperplasia of the parathyroids, followed by degeneration. It would appear that, in the chick, the antirachitic vitamin, whether fed or produced within the animal, is necessary to the proper function of the parathyroids and that, in its absence, there is first hyperplasia and then degeneration.

The hypothesis that such action in increasing the calcium concentration of the serum or in preventing tetany as may sometimes be shown by irradiated ergosterol or by cod liver oil is strengthened by the result of the experiment reported by Hess and Lewis (39). They state:

"In a monkey in which latent tetany was induced and had been maintained for several months by means of a diet low in calcium, large amounts of irradiated ergosterol given by mouth promptly raised the serum calcium to a normal level. After the parathyroid glands had been removed, the calcium fell once more and could not be raised by repeated doses of irradiated ergosterol."

But such considerations do not explain the results originally reported by Jones and corroborated by Brougher and by our experience with Dogs 57, 62, 80, and 83. There were several dogs

that were free from tetany although the concentration of calcium in the serum was below what is generally considered to be the tetany level. When we began this work, we believed that this might be due to diminished concentration of protein in the serum, for Salvesen and Linder (40) had found a low calcium concentration in the protein-poor sera of patients with nephrosis and, in a previous publication, one of us (41) had reported a rather low concentration of calcium (7.4 mg. per 100 cc.) with a low protein content (less than 4.5 per cent) in a dog that had not developed tetany in spite of what was believed to be a complete thyro-parathyroidectomy. Accordingly, in this series of experiments, we have regularly determined the protein content (nitrogen of the trichloroacetic acid precipitate $\times 6.25$) of the sera. It is evident from the results obtained (Table I) that the low calcium content of the sera of these animals not in tetany cannot be due to a diminished concentration of protein. Another explanation must be sought for these findings as well as for the similar low calcium concentrations in latent tetany reported by Salvesen (23) and by Blum (42).

One of us (41) has previously advanced the following considerations: Death in tetany seems to be brought about by exhaustion or by asphyxia caused by a respiratory spasm. The tetany is due to the hyperexcitability which, in turn, is induced by the low concentration of calcium in the body fluids. An attack of tetany can frequently be promoted or intensified by rough handling and we believe that we have sometimes seen a slight tremor disappear as the animal was petted. It seems possible that, if the development of the hyperexcitability or, rather, of its cause, the low concentration of calcium, can be delayed, time is given for the development of inhibitions or of other mechanisms which counteract this hyperexcitability. It may be that some such mechanism controlling the hyperexcitability is responsible for the lesser mortality after parathyroidectomy observed by Hammett (43, 44) in gentled albino rats as compared with ordinary albino rats or wild Norway rats.

Both Jones and Brougher agree that the fall in the concentration of calcium in the serum after thyro-parathyroidectomy was slower in the animals that received cod liver oil than it was in those that did not. Both Jones and we find a tendency for the concentration

of calcium in the serum after a period of cod liver oil feeding to be rather higher than normal, indicating the presence of an increased amount of parathyroid hormone.

TABLE VI.

Effect of Replacing Maize Oil by Cod Liver Oil, upon Excretion of Nitrogen, Phosphorus, and Calcium.

Dog 60.—Female, weight 9.3 kilos. Diet, 20 gm. of dried meat residue, 80 gm. of cracker meal, 35 cc. of maize oil, 12 gm. of infusorial earth, 2 gm. of $\text{Fe}(\text{OH})_3$, and 500 cc. of water. Intake, 4.02 gm. of N, 0.130 gm. of P, 0.013 gm. of Ca. On April 24, and thereafter, cod liver oil replaced the maize oil.

Date.	Urine.			Feces.									
	N	P	Ca	Weight.	SiO ₂	Fe	P	Ca	Per gm. SiO ₂ .		Per gm Fe.		
									P	Ca	P	Ca	
1927	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	mg.	mg.	mg.	mg.	
Apr. 18	3.11	0.050	0.001	26.1	11.65	1.48	0.129	0.186	11.0	15.9	87.5	126	
“ 19	4.00	0.095	0.001	41.3	19.53	2.38	0.196	0.284	10.0	14.5	82.5	120	
“ 20	4.02	0.086	0.001	18.5	9.06	1.18	0.096	0.122	10.6	13.5	81.8	104	
“ 21	3.08	0.078	0.000	12.7	6.02	0.74	0.062	0.082	10.3	13.7	83.2	110	
“ 22	3.62	0.069	0.000	17.8	8.64	1.04	0.092	0.116	10.6	13.5	88.4	112	
“ 23	3.77	0.052	0.000										
Average.	3.60	0.072	0.0005	19.4	9.15	1.13	0.096	0.132	10.5	14.4	84.5	116	
Apr. 24-26	9.03	0.148	0.002	71.3	35.4	4.30	0.362	0.557	10.2	15.8	84.1	130	
Apr. 27-29	9.10	0.192	0.001	57.3	29.5	3.43	0.294	0.476	10.0	16.1	85.8	139	
Apr. 30-May 2	10.22	0.220	0.002	47.2	23.8	2.65	0.252	0.407	10.6	17.2	94.9	153	
May 3-5	11.53	0.250	0.004	59.4	29.3	3.32	0.324	0.498	11.1	17.0	97.6	160	
Average per day.	3.32	0.0675	0.0007	19.6	9.83	1.14	0.103	0.161	10.4	16.4	89.9	141	

Additional evidence for the stimulation of the parathyroids as a result of the administration of cod liver oil may be found in the results of an experiment upon Dog 60 (Table VI). This animal had been upon a calcium-poor diet since March 3, 1927. This consisted of 20 gm. of dried meat residue, 80 gm. of cracker meal, 35 cc. of maize oil, 12 gm. of infusorial earth, and 2 gm. of $\text{Fe}(\text{OH})_3$.

It contained 4.02 gm. of nitrogen, 0.130 gm. of phosphorus, and 0.013 gm. of calcium. In a series of experiments, with which we are not at present concerned, the infusorial earth was replaced by kaolin and then again employed, and the maize oil was then replaced by an isodynamic quantity of lactose. Beginning with April 15, the original diet was resumed. On April 24, the maize oil was replaced by cod liver oil. As is evident from the figures in Table VI, the excretion of calcium was promptly increased, though that of nitrogen was diminished. The excretion of phosphorus remained almost unchanged but the small rise that might be expected to accompany the increase in the calcium output was masked by the drop in the phosphorus excretion due to the diminished protein metabolism. If allowance be made for this, it is found that the increase of approximately 29 mg. in the daily excretion of calcium was accompanied by an increase of approximately 15 mg. of phosphorus. This is, within the limit of error of such determinations, the ratio in bone and in the increased excretion brought about by the administration of parathyroid extract (Greenwald and Gross (4, 5), Hunter and Aub (6)).

It is true that the result of this experiment is not in accord with those reported by Sjöllema (45) or by Yoder (46). In our opinion, the conclusions of both these authors are of doubtful general validity. In Sjöllema's experiments, on rabbits, the food intake was not constant and the diets employed were not well suited for the purpose. They produced a strongly acid urine, abnormal for a rabbit, and the so called calcium-rich diet, furnishing about 18 mg. of calcium per kilo of body weight, contained about 7 times as much phosphoric acid as calcium. The calcium-poor diet, furnishing as it did 7 mg. of calcium per kilo of body weight to one rabbit and 14 mg. to the other and containing 20 times as much phosphoric acid as calcium, seems to have been not so much calcium-poor as phosphorus-rich.

Yoder claimed that the utilization of calcium was better in rats, weighing about 150 gm., that received cod liver oil or irradiation than it was in those without such treatment. But the data presented are very irregular and quite extraordinary. The only really significant differences appear to have been at the end of 30 days of the experiment. At that time, the control animals showed an apparent negative utilization of an average -112

per cent for calcium and of -83 per cent for phosphorus. This means that more than twice as much calcium and almost twice as much phosphorus were being excreted as were fed. Yoder does not state how much food his rats ate but according to Karelitz and Shohl (47), rats weighing 45 gm. ate 4.4 gm. per day of a ration which, except for the addition of 10 per cent of lard, was identical with that employed by Yoder. We may, therefore, safely conclude that Yoder's rats, weighing 150 gm., consumed at least 12 gm. of the unmodified ration per day. This would mean an intake of 0.144 gm. of calcium per day and a negative utilization of 112 per cent would mean a loss of 0.16 gm. of calcium per day. This seems to us to be a totally incredible amount. When we consider also that even the animals receiving cod liver oil or irradiation also showed negative utilizations, we feel justified in regarding Yoder's results as probably due to some extraneous influence or to some error in technique.

That the administration of cod liver oil promotes calcium assimilation in *young* rats seems abundantly proved by the work of Park, Guy, and Powers (48), Schultzer (49), Bergcim (25), and Shohl, Bennett, and Weed (50). Similar results were obtained in a young dog by Shohl and Bennett (51), in infants with tetany by Liu (52), and in those with rickets by Daniels, Stearns, and Hutton (53).

There is some indication that there is an optimal amount of cod liver oil and that an excess may be less effective in promoting calcium assimilation than is a smaller quantity. Thus, Tso, Yee, and Chen (54) observed a smaller retention of both calcium and phosphorus in a child when it received 15 cc. of cod liver oil than when it received only 10 cc.

There seem to have been but few experiments on the effect of the administration of cod liver oil on calcium assimilation in adult animals. Liu (55) found that it did not increase the concentration of calcium in the blood in a case of adult tetany. Meigs, Turner, Harding, Hartman, and Grant (56) reported that it did not improve calcium assimilation in a lactating cow but their experiments have been adversely criticized by Hart, Steenbock, Kletzien, and Scott (57). The authors last named found that the administration of the non-saponifiable fraction of cod liver oil to lactating goats improved calcium assimilation but their experi-

ments with cod liver oil were inconclusive. Havard and Hoyle (58) found that the ingestion of irradiated ergosterol by men increased neither the inorganic phosphorus content of the blood nor the calcium content of the serum.

It is quite likely that additional metabolism experiments would yield additional apparently contradictory results. Calcium is absorbed from the small, and excreted into the large intestine. The extent of both of these processes must be determined by certain equilibria affecting the concentration of diffusible calcium in the intestine and in the plasma. If we assume that the parathyroid hormone, directly or indirectly, increases the calcium-dissolving power of the plasma and that cod liver oil stimulates the parathyroids, it may readily be seen that the addition of cod liver oil to a diet rich in calcium that is being fed to young animals, in which calcium deposition is rapid, or to lactating animals, in which also large amounts of calcium are removed from the blood, might lead to an increased absorption of calcium; whereas the same addition, particularly when combined with a calcium-deficient diet, in the case of adult and non-lactating animals, in which there is no biologically imperative demand for calcium, would lead to an increased excretion of calcium.

Naturally, this explanation applies more directly to experiments in which parathyroid extract is administered. It may serve to reconcile the differences between the results of the authors (4, 5) and of Hunter and Aub (6) on the one hand, and those of Robinson, Huffman, and Burt (59),⁷ Liu (52, 55) and Hoag, Rivkin, Weigele, and Berliner (60), on the other, as well as the variations in the results reported by the authors last named.

In the case of thyroparathyroidectomized dogs, the combination of a large store of parathyroid hormone and of more active parathyroid tissue, some of which is not removed, may well keep the concentration of calcium in the plasma from falling too rapidly. For some reason, Jones was more successful in achieving this result than others have been. Brougher found a lesser degree of protection, Wade still less, and we least of all. It seems likely

⁷ In our opinion, Robinson, Huffman, and Burt employed periods that were much too short and diets that contained so much calcium that increases in the excretion of the order we observed in dogs after the administration of parathyroid extract would be entirely masked.

that the amount and character of the food ingested, before and, particularly, after the operation was responsible for this variation. When the dogs refused to eat, Jones and Wade allowed them to fast; Brougher administered milk; we gave the same diet as before the operation, using the stomach-tube.

In any event, the line between tetany and no tetany is a very narrow one. From the results reported by Salvesen and from those obtained with Dogs 62 and 80 of this series, it seems that as small a difference as 0.5 mg. of calcium per 100 cc. of plasma may determine whether the dog will behave in quite normal fashion or exhibit severe tetany.

SUMMARY.

An attempt was made to study, by means of metabolism experiments, the method by which the administration of cod liver oil before thyroparathyroidectomy protects dogs against the occurrence of tetany. It was found that such protection was much less frequently attained than has been claimed by others. In fact, our experiments, considered by themselves, would have led us to conclude that cod liver oil has no protective action. These differences and the results of our metabolism experiments are analyzed and an explanation is offered.

It is suggested that:

1. Almost all dogs possess accessory parathyroid tissue which is not removed in the course of the ordinary thyroparathyroidectomy.

2. Tetany, following parathyroidectomy, is due, not only to the low concentration of diffusible calcium in the plasma but also to the rapidity with which this low concentration is attained. If the diminution is made sufficiently slowly, the animal may adjust itself to such low level of plasma calcium.

3. The administration of cod liver oil stimulates parathyroid tissue to increased activity. Therefore, after ordinary thyroparathyroidectomy, animals that have previously been treated with cod liver oil are left with a larger store of parathyroid hormone and with more active accessory parathyroid tissue than are animals that have not been so treated. Consequently, the concentration of calcium in the plasma falls more slowly and acute tetany is avoided or delayed. The organism is given a longer time in

which to adjust itself to the low concentration of plasma or the remaining parathyroid tissue has a better opportunity to hypertrophy sufficiently to supply the animal's needs. Possibly, both processes are involved.

4. In an experiment upon a dog on a low calcium diet, a change from maize oil to cod liver oil led to an increased excretion of calcium.

5. The opinion is expressed that the effect of cod liver oil, and of the administration of parathyroid extract, upon calcium metabolism will be found to vary with the calcium content of the diet, the age of the animal, and the need for calcium in the organism. Certain observations in the literature supporting such a view are cited.

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THE PREVENTION OF THE TETANY OF PARATHYROIDECTOMIZED DOGS.

II. LACTOSE-CONTAINING DIETS.

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In 1923, Dragstedt and Peacock (1) reported that they had been able to prevent the appearance of tetany in thyroparathyroidectomized dogs by the feeding of large amounts of lactose. Apparently, they found dextrin to have a similar action for they stated (p. 432): "Starvation does not prevent the appearance of tetany nor do diets rich in other carbohydrates than lactose and dextrin." However, in the experiments described in their paper, only lactose was used.

There were three groups of these experiments. In the first of these, dogs from 2 to 3 months old received a diet of white bread and whole milk, *ad libitum*, with 60 gm. of lactose every day; in the second, adult dogs, 2 to 4 years old, received a daily diet of 500 gm. of boiled rice, 100 gm. of beef heart, and 50 gm. of lactose; in the third, nineteen adult dogs, 2 to 4 years old, received a diet of white bread and skim milk, *ad libitum*, with 50 to 125 gm. of lactose per day. The amount of milk ingested daily was not more than a liter and was generally less than half of that. In the first two groups, thyroparathyroidectomy was performed after 4 to 7 days of such feeding; in the third group, not until after a longer period (7 to 21 days).

The animals of the first group survived from 4 to 10 days, the average being 7.66 days. None showed tetany. Of the eleven animals in the second group, all but three developed tetany in from 2 to 5 days. One of those not showing tetany died of pneumonia on the 5th day after the operation, leaving two without any obvious signs of parathyroid insufficiency. Of the eight dogs in which tetany did develop, one died of pneumonia, two in tetany, two in depression (9 days after the operation), and one of distemper (7 days after the operation). The other two recovered from the tetany and were still alive at the time the report was made.

Of the third group of nineteen adult dogs, all but two developed tetany but only two died within 4 days and these were pregnant animals. Eleven

recovered from the tetany and continued in good condition for months. Four of these died in tetany in from 4 to 9 months, apparently after the lactose diet had been discontinued. After 6 weeks of the lactose diet, the four animals of the second group and the seven of the third that had survived were able to take the usual laboratory diet of meat and vegetables without developing tetany.

Dragstedt and Peacock regarded the results of these experiments as indicating that lactose-containing diets protected parathyroidectomized dogs against tetany and believed this effect to be due to the change in the intestinal flora from the normal, predominatingly proteolytic, to one predominatingly aciduric. "Nevertheless," they stated, "the addition of milk to the diet seems to have a definite beneficial influence." (p. 433) But they believed this beneficial influence of milk to be relatively slight.

In spite of the explicit statement that other carbohydrates, except dextrin, do not protect against tetany, no experiments are reported.

It is, we believe, the experience of most workers in this field that about one dog in ten will not develop tetany after the usual thyroparathyroidectomy or will recover spontaneously, without the use of any medication or of a special diet. Of the dogs not receiving milk in the experiments of Dragstedt and Peacock, only two out of eight recovered. This ratio does not seem to us to be sufficiently great to warrant the conclusion that lactose-containing diets prevent tetany.

Inouye (2) reinvestigated the protective action of lactose and also employed other carbohydrates. He found that dextrin diets did not prevent tetany but he was successful in preventing tetany by feeding diets containing lactose, or lactose and sucrose, or galactose and sucrose. He also found that casein, the sole protein employed, and lactose were antagonistic, that is, that an increase in the casein content of the diet necessitated an increase in the lactose content in order to preserve its protective action.

All but three of the dogs received bone ash, but Inouye believed that this could have played no significant part because the three dogs that received Cellu Flour, a cellulose preparation, instead of bone ash, were also protected from tetany by lactose diets.

However, Inouye neglected to consider the calcium content of

the salt mixture used. 1.2 gm. of a mixture containing 4 parts in 19 of calcium lactate were added to each 100 gm. of food. Therefore, this amount of food, yielding 480 calories, contained 32.8 mg. of calcium. Inouye does not state how much food his dogs received, but Gross (3), who used the same kind of diet, found that 80 calories per kilo were needed. Therefore, one is justified in assuming that Inouye's dogs received about 5.5 mg. of calcium per kilo of body weight from the salt mixture alone.

The calcium content of the casein employed, which was, apparently, not determined, may also have been of some significance. We have analyzed three different specimens of commercial casein. Two of them contained only 0.0002 per cent but the third contained 0.2 per cent. The dogs in question received diets containing 5.5 per cent casein. If this had contained 0.2 per cent calcium, 100 gm. of the food, furnishing 480 calories, would have contained 11 mg. of calcium. In this manner, the dogs would have received an additional 2 mg. of calcium per kilo of body weight. The preparation of casein used by Inouye may have contained less calcium but it may have contained more. It is greatly to be regretted that the calcium content of the diet was not accurately determined.

Inouye also studied the calcium and inorganic phosphorus content of the sera of his animals. He stated (p. 534):

"When the animals were kept free from manifest tetany by a lactose diet, the calcium content of blood serum at first showed a value slightly lower than normal, usually about 8 to 9 but never below 7 mgm., and this slightly lower value showed a tendency to rise gradually to normal in the course of time after the operation. On the other hand the phosphorus content of the blood serum in thyroparathyroidectomized dogs was always increased, even if the animals were kept free from tetany by lactose feeding or by calcium administration. This increased phosphorus [content] returned gradually to normal in the course of time."

The method by which lactose diets may prevent tetany was next studied by Gross (3). He reported on only two animals, both of which he stated "were continuously on the border-line of tetany" in spite of an intake of nearly 20 gm. of lactose per kilo per day. He concluded from his experiments that "in parathyroidectomized dogs lactose prevents the retention of phosphorus and calcium."

Unfortunately, even considerable retention of these elements could have occurred and yet have been entirely masked by the large amounts ingested and eliminated. The diet employed by Gross contained bone ash, resulting in an intake of 12.65 gm. of CaO and of 12.70 gm. of P_2O_5 in each 5 day period by dogs weighing about 12 kilos. This is at the rate of approximately 0.2 gm. of CaO or P_2O_5 per kilo per day.

Except when large amounts of calcium lactate, carbonate, or chloride were administered after a long period on a calcium-poor diet, the highest retentions of calcium we have observed after thyroparathyroidectomy have been approximately 7 mg. per kilo per day.¹ With an intake and excretion of more than 20 times as much, a retention of this magnitude may easily have been concealed.

The phosphorus retentions we have observed and which affected only the urine have been somewhat greater, but in none of our experiments do they seem ever to have been greater than approximately 30 mg. per kilo per day and they were usually only half as great. But a retention of 15 mg. of phosphorus, or 34 mg. of P_2O_5 , per kilo per day should have been detectable in the experiments of Gross.

As a matter of fact, study of the figures reported does reveal evidence of retention of both phosphorus and calcium. In one of the experiments (Gross,*Table II), the excretion of P_2O_5 in the urine of the 5 days following parathyroidectomy was less by 0.620 gm. than in the preceding period. In this experiment, the nitrogen excretion was slightly increased after the operation. In the other experiment, also, the P_2O_5 excretion in the urine was decreased but the nitrogen excretion was also diminished, so that the ratio of P_2O_5 : N was not appreciably altered. But, in this experiment, the effect of the parathyroidectomy was complicated by the simultaneous change from a sucrose to a lactose diet. A similar change in one of the control experiments, though not in the other, was accompanied by an *increase* in the P_2O_5 : N ratio.

This was also the case with the calcium excretion. In the experiment in which the animal was on a lactose diet before the

¹ In an experiment reported in the preceding paper of this series (4) a retention of approximately 9 mg. of calcium per kilo per day was observed.

TABLE I.

Summary of Experiments upon Thyroparathyroidectomized Dogs Receiving Lactose-Containing Diets.

Dog No.	Lactose per kilo.	Days until tetany.	Intake.			Daily excretions.							
			N	P	Ca	Before operation.				After operation.			
						days	N	P	Ca	days	N	P	Ca
	gm.		gm.	gm.	gm.		gm.	gm.	gm.		gm.	gm.	gm.
61	10	1	3.82	0.106	0.014	3	3.39	0.167	0.095	1	3.52	0.059	0.090
69	13.6	2	2.61	0.169	0.013	7	2.67	0.202	0.063	2	4.02	0.144	0.110
						1	2.93	0.233	0.103*				
72	12.5	2	2.93†	0.361†	0.022	7	2.39	0.356	0.098	2	2.49	0.122	0.070
74	7.5	2	3.63†	0.436†	0.228	7	4.49	0.483	0.216	2	4.06	0.266	0.224†
76	8.4	3	2.02	0.359	0.297	8	2.25	0.312	0.170	5	2.99	0.250	0.232
77	8.5	5	2.55	0.334	0.415	4	2.48	0.256	0.245	5	2.60	0.210	0.250
						5§	2.63	0.224	0.262	Last 4	2.45	0.161	0.230
79	8.8	3	3.77	0.711	0.710	6	3.51	0.705	0.559	6	4.78	0.646	0.717
						7	3.99	0.699	0.631	Last 5	4.41	0.641	0.649
82	10.3		2.81	0.562	0.697	7	4.57	0.591	0.561	9	7.71	0.725	0.592

* See text, p. 538.

† These figures are only approximately correct. The beef heart used was not analyzed. The figures are based upon analyses of other samples.

‡ See Table III.

§ See Table IV.

|| See Table V.

Dog 61.—Female, weight 6.2 kilos. Beginning 25 days before the operation the animal was fed a diet of lactose 63 gm., meat residue 25 gm., cracker meal 30 gm., infusorial earth 10 gm., Vegex 1 gm., water 350 cc. N determined on urine only. Dog found dead 47 hours after operation.

Dog 69.—Male, weight 9.1 kilos. Beginning 15 days before the operation the animal was fed a diet of lactose 125 gm., Shredded Wheat 40 gm., meat residue 15 gm., water 500 cc. Very severe tetany 50 hours after operation.

Dog 72.—Male, weight 10.0 kilos. Beginning 22 days before the operation the animal was fed a diet of lactose 125 gm., hashed beef heart 60 gm., Shredded Wheat 70 gm., infusorial earth 10 gm., water 400 cc. Severe tetany 48 hours after operation. Relieved by NH_4Cl . Died in tetany after NH_4Cl was discontinued.

Dog 74.—Female, weight 10.5 kilos. Beginning 13 days before the operation the animal was fed a diet of lactose 70 gm., dry milk 20 gm., Shredded Wheat 35 gm., hashed beef heart 70 gm., water 400 cc. Tetany 60 hours after operation. Severe tetany 72 hours after operation; relieved by NH_4Cl , but tetany returned after NH_4Cl was discontinued.

Dog 76.—Female, weight 10 kilos. Beginning 20 days before the operation

TABLE I—*Concluded.*

tion the animal was fed a diet of lactose 72 gm., dry milk 26 gm., Shredded Wheat 45 gm., NaCl 1 gm., Fe(OH)₃ 3 gm., water 300 cc. Tetany at intervals on 3rd and 4th days; severe on 5th day; relieved by NH₄Cl. Recurred when administration of NH₄Cl ceased.

Dog 77.—Female, weight 10.5 kilos. Beginning 12 days before the operation the animal was fed a diet of lactose 72 gm., dry milk 36 gm., cracker meal 45 gm., NaCl 1 gm., Fe(OH)₃ 3 gm., water 400 cc. Tetany relieved by NH₄Cl on 6th day. Recurred when administration was discontinued. Again relieved by NH₄Cl only to recur when NH₄Cl was no longer given.

Dog 79.—Male, weight 12 kilos. Beginning 28 days before the operation the animal was fed a diet of lactose 80 gm., dry milk 56 gm., Shredded Wheat 16 gm., salt mixture² 3.75 gm., beef heart 25 gm., Vitavose 10 gm., water 400 cc. Twitching on 3rd day. Slight spasms on 5th day. Otherwise no sign of tetany.

Dog 82.—Male, weight 8.1 kilos. Beginning 11 days before the operation the animal was fed a diet of lactose 57 gm., dry milk 57 gm., salt mixture 2.5 gm., water 600 cc. Slight tetany at intervals on 3rd to 7th days. Gradually lost weight to 19th day; when killed weighed 5.2 kilos.

operation, there were negative balances of 1.00 and 0.180 gm. of CaO, respectively, in the two periods preceding the operation. During the next four 5 day periods all the balances were positive, the figures being 0.524, 0.645, 0.160, and 0.077 gm., respectively. In the other experiment, the effect seems to have been just the opposite (the signs in the table should be reversed), a positive balance being changed to a negative one. Later, the balance again became positive. But in this experiment, the operation was accompanied by a change in diet. A similar change in diet, without operation, was, in one of the control experiments, followed by a similar change of a positive into a negative balance, and, in this case, the balance remained negative in the second 4 day period.

It is, therefore, quite evident that the conclusions Gross drew from his experiments are quite unwarranted and, probably, erroneous.

It seemed to be advisable to reinvestigate the manner in which lactose diets prevent tetany. Accordingly, we began a series of experiments, the results of which are summarized in Table I. It was found rather difficult to induce the dogs to take food regularly which contained large amounts of lactose, and various

² The salt mixture was that described by Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923).

materials were added in order to secure regular consumption of the food. In every experiment but the first, the amount of lactose fed was sufficiently large to secure liquid feces.

The first three animals received diets poor in calcium but containing 10, 13.7, and 12.5 gm., respectively, of lactose per kilo of body weight. All of these animals developed severe tetany in less than 3 days after operation. In the following experiments,

TABLE II.

Analyses of Sera of Dogs on Lactose Diets before and after Thyroparathyroidectomy.

Dog No.	Before.			After.				Remarks.
	Protein.	Ca.	Inorganic P.		Protein.	Ca	Inorganic P.	
	per cent	mg. per cent	mg. per cent	days	per cent	mg. per cent	mg. per cent	
69	5.48	10.9	5.48	2	5.31	6.35	6.19	Tetany.
72	4.35	9.9	6.37	2	4.41	7.19	7.81	"
76	5.50	11.8	6.40	3	5.03	6.40	7.33	Beginning tremor.
77	5.63	11.0	5.55					
79	7.65	9.75	3.90	3	6.51	5.49	4.48	Slight twitching.
				5	6.61	6.01	4.29	No twitching except when tube was passed.
				7	5.94	5.92	5.31	No twitching.
82	5.61	10.1	3.22	2	5.31	6.28	5.37	" "
				3	5.01	5.43	5.39	Tetany appeared shortly after sample was drawn.
				5	4.39	4.68	6.77	Tetany.
				19	4.41	5.53	5.40	No twitching in last 10 days.

we fed diets containing increasing amounts of calcium, in the form of dry milk. It was found that, although the calcium intake was increased to as much as 0.7 gm. per day, tetany was not entirely prevented. It was, however, diminished in severity in those animals that received the larger amounts of dry milk. Four dogs, Nos. 72, 74, 76, and 77 were treated with ammonium chloride after the appearance of tetany. The tetany disappeared only to recur in a day or two, *although the lactose diet was continued.* In one

dog, No. 74, the experiment was repeated; tetany again disappeared, only to recur when the ammonium chloride was discontinued.

The two dogs receiving the largest amounts of calcium in their food recovered from the tetany spontaneously. One was killed on the 7th day after the operation. The other was allowed to live, though it was obviously not in good health, until 19 days after the operation.

In Table II there are collected the results of the analyses of the sera at the time of the operation and at intervals thereafter. In every case there was a fall in the concentration of calcium and an increase in that of inorganic phosphorus. In the sera obtained after spontaneous recovery from tetany, the concentration of calcium was below the tetany level but the concentration of proteins was also low, so that the concentration of diffusible calcium was probably not diminished as much as was that of total calcium. Nevertheless, it must be recognized that in the sera of both Dogs 79 and 82, the relation between calcium, inorganic phosphorus, and protein, was, finally, such as would generally be expected to give rise to tetany, although this was absent. Both of these animals were sick and the failure of tetany to appear may have been due to the depression accompanying the general weakness, etc. But it is possible that these dogs presented instances of adaptation to low calcium concentration, such as have been reported by others and are discussed by us in the preceding paper of this series (4).

The excretion of phosphorus was, in every case but one, diminished after thyroparathyroidectomy (Table I). In this one experiment, there was a marked increase in the excretion of nitrogen, probably due to an infection. The ratio P:N in the urine was regularly diminished, as we have always found it to be. But there was no such consistent and regular retention of calcium as we have observed in all of our previous experiments. Curiously enough, the diminished excretion of calcium was observed in two (Dogs 61 and 72) out of the three dogs that had been losing calcium prior to the operation. In the third dog (No. 69), there seems to have been a sudden increase in the calcium excretion on the day before the operation. If whatever was responsible for this increase continued to act in the experimental

period, the increase due to it could easily have masked the usual calcium retention.

In Tables III to VII, we have presented in detail the results of the analyses of the mixed urine and feces of the other dogs of this series. Examination of these appears to be of interest.

Dog 74 (Table III) was in calcium equilibrium during the week before the operation. Material for analysis was secured for only 2 days thereafter and this showed a continuance of the excretion of calcium at the same level.

TABLE III.

Dog 74.—Female, weight 10.5 kilos. Diet, 70 gm. of lactose, 20 gm. of dry milk, 35 gm. of Shredded Wheat, 70 gm. of hashed beef heart, 400 cc. of water, since March 6. Intake, 3.63 gm. of N., 0.436 gm. of P, 0.228 gm. of Ca.

Date	Excretion in combined urine and feces					Remarks
	N	P	Ca	K	Na	
1928	gm	gm	gm	gm	gm	
Average, Mar. 12-18.	4.49	0.483	0.216	0.698	0.211	Control period
Mar. 19	1.05	0.153	0.210	0.098	0.192	Thyroparathyroidectomy.
" 20	8.06	0.378	0.238	0.835	0.218	
" 21	3.12	0.179	0.007	0.714	0.224	No feces Tetany, very severe, at close of period
Average, Mar. 19-21.	4.08	0.237	0.152	0.549	0.211	
Average, Mar. 19-20.	4.06	0.266	0.224	0.465	0.205	

In the case of Dog 77 (Table IV), it is obvious that some urine was carried over from the preoperative to the postoperative period. If the first postoperative day be included in the fore period and not in the after period, the calcium excretion shows a slight decrease. If this is not done, it remains practically unchanged.

Demarcation of the periods in the experiment on Dog 79 (Table V) failed because of retention of feces. Consequently, the more correct value for the calcium excretion is obtained by including the first postoperative day with the fore period. If this

is done, the calcium excretion again shows practically no change as a result of the operation.

Finally, there remain two experiments, those on Dogs 76 and 82 (Tables VI and VII), in which the excretion of calcium appears to have been *increased* after thyroparathyroidectomy. However, in neither of these was there a loss of calcium from the body.

TABLE IV.

Dog 77.—Female, weight 10.5 kilos. Diet, 72 gm. of lactose, 36 gm. of dry milk, 45 gm. of cracker meal, 1 gm. of NaCl, 3 gm. of Fe(OH)₃, 400 cc. of water, since March 29. Intake, 2.55 gm. of N, 0.334 gm. of P, 0.415 gm. of Ca.

Date.	Excretion in combined urine and feces.				Remarks.
	N	P	Ca	Mg	
<i>1928</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Apr. 5	2.82	0.360	0.274	0.065	Apparently retained urine.
" 6	2.50	0.273	0.285	0.058	
" 7	2.88	0.252	0.252	0.059	
" 8	1.73	0.137	0.168	0.016	
Average.	2.48	0.256	0.245	0.050	
Apr. 9	3.23	0.199	0.331	0.085	Thyroparathyroidectomy.
Average, Apr. 5-9.	2.63	0.224	0.262	0.057	
Apr. 10	2.63	0.088	0.202	0.110	Tetany, Apr. 14, a.m.
" 11	2.42	0.134	0.250	0.092	
" 12	2.35	0.224	0.256	0.069	
" 13	2.39	0.197	0.214	0.127	
Average, Apr. 9-13.	2.60	0.210	0.250	0.097	
Average, Apr. 10-13.	2.45	0.161	0.230	0.100	

There was merely a lessened retention and, in the case of Dog 82, the change was rather slight. In both dogs there was, after the operation, a marked increase in the excretion of nitrogen, leading to an increase in the already existing nitrogen deficit. In the case of Dog 82, this increase in the excretion of nitrogen was very large, far greater than the increase in that of calcium. In the

other experiment there was no such disproportion but the ratio of the increase in the excretion of calcium to that in the excretion of nitrogen was nevertheless, considerably less than that in the

TABLE V.

Dog 79.—Male, weight 12.0 kilos. Diet, 80 gm. of lactose, 56 gm. of dried milk, 16 gm. of Shredded Wheat, 25 gm. of beef heart, 10 gm. of Vitavose, 3.75 gm. of salt mixture,* 400 cc. of water, since April 30. Intake 3.99 gm. of N, 0.770 gm. of P, 0.710 gm. of Ca.

Date.	Excretion in combined feces and urine.				Remarks.
	N	P	Ca	Mg	
1928	gm.	gm.	gm.	gm.	
May 22	4.13	0.881	0.669	0.190	
" 23	3.91	0.814	0.700	0.222	
" 24	3.66	0.711	0.608	0.209	
" 25	3.81	0.789	0.725	0.213	
" 26	2.29	0.527	0.545	0.177	
" 27	3.27	0.505	0.108	0.044	No feces voided this day.†
Average.	3.51	0.705	0.559	0.176	
May 28	6.84	0.669	1.061	0.337	Thyroparathyroidectomy.
Average	3.99	0.699	0.631	0.199	If May 28 is included in fore period.†
May 29	5.36	0.695	0.944	0.241	
" 30	3.03	0.505	0.554	0.179	
" 31	4.83	0.633	0.458	0.165	Twitching
June 1	2.98	0.474	0.611	0.186	
" 2	5.63	0.899	0.686	0.237	Slight spasm of jaws.
Average.	4.78	0.646	0.717	0.224	From May 28 to June 2.
"	4.41	0.641	0.649	0.201	" " 29 " " 2.

* Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923).

† The fact that no feces were voided on May 27 makes it advisable to include the 1st day of the postoperative period in order to secure a more accurate average. Similarly, the average for May 29 to June 2 more accurately represents conditions in the experimental period than does that of May 28 to June 2.

food. In other words, the effect might have been due, not to any specific effect on calcium metabolism, but merely to a failure of the animal to absorb as much food after the operation as before.

TABLE VI.

Dog 76.—Female, weight 10 kilos. Diet, 72 gm. of lactose, 26 gm. of dry milk, 45 gm. of Shredded Wheat, 1 gm. of NaCl, 3 gm. of Fe(OH)₃, 300 cc. of water, since March 27. Intake, 2.02 gm. of N, 0.359 gm. of P, 0.297 gm. of Ca.

Date.	Excretion in combined urine and feces.				Remarks.
	N	P	Ca	Mg	
1928	gm.	gm.	gm.	gm.	
Average, Apr. 8-15.	2.25	0.312	0.171	0.105	Control period.
Apr. 16	3.37	0.207	0.247	0.101	Thyroparathyroidectomy.
" 17	1.71	0.164	0.156	0.079	
" 18	2.34	0.207	0.185	0.084	
" 19	4.02	0.325	0.218	0.111	Tetany.
" 20	3.49	0.346	0.353	0.118	" severe at close of period.
Average.	2.99	0.250	0.232	0.099	

TABLE VII.

Dog 82.—Male, weight 8.1 kilos. Diet, 57 gm. of lactose, 57 gm. of dry milk, 25 gm. of salt mixture, *500 cc. of water, since September 22. Intake 2.81 gm. of N, 0.562 gm. of P, 0.697 gm. of Ca.

Date.	Excretion in combined urine and feces.			Remarks.
	N	P	Ca	
1928	gm.	gm.	gm.	
Average, Sept. 26-Oct. 2.	4.57	0.591	0.561	Control period.
Oct. 3	6.27	0.426	0.405	Thyroparathyroidectomy.
" 4	5.51	0.710	0.765	
" 5	7.46	0.786	0.619	
" 6	8.34	0.650	0.478	Slight twitching.
" 7	9.86	0.877	0.652	" "
" 8	9.39	0.693	0.609	" "
" 9	8.74	0.707	0.320	" "
" 10	6.57	0.848	0.776	" "
" 11	7.21	0.827	0.707	No "
Average.	7.71	0.725	0.592	

* Cowgill, G. R., *J. Biol. Chem.*, **56**, 725 (1923).

However, the possibility of a direct effect on calcium metabolism must be recognized; nor is it at all inconsistent with the hypothesis concerning parathyroid function advanced by the authors. In an animal that is losing calcium, there is a continuous flow of calcium from the bones to the intestine. After removal of the parathyroids and consequent failure of the parathyroid hormone and calcium-dissolving substance, the plasma cannot carry the usual amount of calcium and excretion at once falls. However, in an animal that is on a calcium-rich diet and that is storing large amounts of calcium, failure of the parathyroid hormone may well lead to a lowering of the rate of this transport of calcium from the intestine to the bones or other tissues.

It would be a matter of great interest to repeat these experiments with diets containing more nitrogen and maintaining nitrogen equilibrium, and on animals subjected only to parathyroidectomy. Since we are no longer able to continue this work, we hope that others may do so.

There is, of course, in the experiments here reported no support for the view that lactose diets protect against tetany solely by virtue of the change in the intestinal flora that they induce. The preliminary periods in our experiments were from 10 to 28 days in length and the average time in those experiments in which liquid feces were secured was 17 days. The only experiments in which even a slight degree of protection was achieved were those in which the diet contained so much calcium that a positive balance was continuously maintained. It would seem that such protective action as lactose-containing diets may exert is due entirely to the fact that these make more available the calcium of the diet. Such action has been reported by Irving and Ferguson (5) and by Bergeim (6). This increased availability may be due to a change in the intestinal flora. To that extent, and to that extent only, does the evidence support the views of Dragstedt and Peacock. The only experiments that are not in entire agreement with this hypothesis are the three experiments of Inouye to which reference has already been made. In these experiments, the calcium content of the diet was not altogether negligible and may have been considerable.

SUMMARY.

Lactose-containing diets protect dogs against tetany only if large amounts of calcium are also contained in the diet. It is believed that the lactose acts by making the calcium more available to the animal. Metabolism studies showed the usual retention of phosphorus after thyroparathyroidectomy but, *on high calcium diets*, the retention of calcium seemed to be decreased. Since the nitrogen excretion was also increased, the significance of the increased calcium excretion is not unequivocal. In any case, it is believed not to be inconsistent with the hypothesis that the parathyroid hormone, directly or indirectly, increases the solubility of calcium in the blood plasma and other body fluids.

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THE COLORIMETRIC DETERMINATION OF THE SERUM PROTEINS.

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In connection with the study of the state of the calcium of the blood, we desired a rapid method for determining serum proteins. Wu (1) and Wu and Ling (2) have published a method for the determination of plasma proteins based on the color developed with Folin's phenol reagent. The method as described by these authors is not very satisfactory due to the formation of turbid precipitates and the slow development of the color. Very recently Folin and Ciocalteau (3) have improved the phenol reagent by the addition of lithium sulfate. This permits the use of greater amounts of phenol reagent and sodium hydroxide without any resulting turbidity. By using more of the reagent the color development takes place far more rapidly and the amount of color is markedly increased. In connection with this improvement in the phenol reagent it seemed to us that a simplification could be obtained in the determination of serum proteins by adopting essentially Howe's method (4) of salting out the globulin with sodium sulfate solution. The sodium sulfate was found to have almost no effect on the color developed so the albumin can be determined directly on an aliquot of the filtrate. We were able in this way to work out a satisfactory method for the analysis of the albumin and globulin, making use of 0.5 cc. of serum.

Reagents.—The reagents required are 22.5 per cent sodium sulfate solution prepared from the anhydrous salt, 5 N sodium hydroxide, a standard tyrosine solution containing 200 mg. of pure dry tyrosine in 1 liter of approximately 0.1 N hydrochloric acid, and Folin's phenol reagent. To keep all the sodium sulfate in solution, it is necessary to keep this reagent in an incubator.

Analytical Procedure.—Pipette 0.5 cc. of serum from a calibrated pipette into a 15 or 20 cc. test-tube. Add exactly 9.5 cc. of 22.5 per cent sodium sulfate solution with a pipette of that volume or from a burette. Agitate thoroughly and set aside for about 2 hours in an incubator at 37° to allow coagulation of the globulin. At the end of this period, filter into another test-tube, using a fairly retentive filter paper (Whatman No. 42 is satisfactory). Examine to see that the filtrate is clear, if not, pour back on the filter paper. After the filtering is nearly complete, remove the tube containing the filtrate to be used for albumin analysis. The residue of globulin in the test-tube in which the precipitation was carried out is now washed onto the filter paper by two washings with 3 cc. each of sodium sulfate solution.

The globulin precipitate is then washed twice more with 3 cc. portions of sodium sulfate solution. The two protein fractions are now estimated according to the following procedure.

Globulin.—The funnel and filter paper containing the globulin precipitate are transferred to a 50 cc. volumetric flask, a small hole is punched in the bottom of the filter paper with a wire or drawn out glass rod, and the globulin dissolved and washed into the volumetric flask by a stream of approximately 0.01 N sodium hydroxide from a wash bottle. The washing is completed with distilled water until the flask is about half full. The filter paper is then unfolded and examined to see that all the globulin is dissolved. To the flask there are now added 2 cc. of 5 N NaOH and 3 cc. of the phenol reagent. The flask must be agitated while the phenol reagent is being added to prevent a large local excess which may result in a turbid precipitate. The flask is now filled to the mark with distilled water and the contents thoroughly mixed. A standard is prepared at the same time by pipetting 4 cc. of the standard tyrosine solution into another 50 cc. volumetric flask, adding about 25 cc. of water, then 2 cc. of sodium hydroxide, and 3 cc. of phenol reagent, agitating the contents of the flask during the course of adding the reagents, and finally filling with water to the graduation mark. After this has stood for from 5 to 10 minutes to insure full color development, the standard is set at the 20 cc. mark on the colorimeter and the unknown compared against it.

Albumin.—For the determination of the albumin, a 5 cc. aliquot

of the filtrate is pipetted into a 50 cc. volumetric flask, about 25 cc. of water are added, then 2 cc. of 5 N sodium hydroxide and 3 cc. of the phenol reagent. The flask is then filled with distilled water to volume and the contents thoroughly mixed. A standard is prepared at the same time with 4 cc. of standard tyrosine solution. After 5 to 10 minutes the colors are read with the standard set at the 20 mm. mark on the colorimeter.

Total Serum Protein.—If no upset of the normal albumin to globulin ratio is suspected, determination of total serum protein, may in many cases, be sufficient. This can be carried out according to the procedure given above, with either 0.2 cc. of whole serum or perhaps more accurately a 2 cc. aliquot of 1 cc. of serum that has been diluted in a 10 cc. volumetric flask. The comparison is carried out in the usual manner against either 4 or 5 cc. of standard tyrosine solution. This determination of total protein gives the possibility of determining the globulin, not directly, but by subtracting the value of the albumin fraction from the total protein. It is more accurate however to determine both albumin and globulin separately as described above.

DISCUSSION.

Some precautions need to be observed to obtain correct analytical results. The color obtained with the phenol reagent is dependent on the age of the serum sample. The amount of color obtained decreases with time as the serum stands. On this account freshly obtained sera that have stood no longer than overnight should be used for the analysis. The alkali and phenol reagent are to be in the proportions recommended. These amounts were found to give optimum color development under the conditions of the analytical method. In tests, made on the standard tyrosine solution, with the above amounts of alkali and phenol reagent, there was obtained a proportionality of color to within 1 per cent, when a solution containing 4 cc. of tyrosine standard was compared against solutions containing 3 and 5 cc. of tyrosine standard. When a similar test, with 2 cc. of alkali and 2 cc. of phenol reagent, was carried out, the solution with 3 cc. of tyrosine standard gave 5 per cent more than proportional color while the 5 cc. tyrosine sample gave 6 per cent too low a color.

Howe (4) found that the salting out of the globulin was constant

when the sodium sulfate concentration was kept between 21 and 22 per cent and the dilution between 10 and 30 volumes. In his micro method (5) 0.5 cc. of serum is diluted to 15 cc. with salt solution. In our procedure it is more convenient to keep the volume down to 10 cc. That there is no objection to changing from the dilution used by Howe to that employed by us is shown by Howe's findings and also by some tests carried out by us. In agreement with Howe, it was found that the same amount of globulin, as shown by the color obtained, was salted out on a 10-, 20-, or 30-fold dilution of the serum.

The effect of sodium sulfate on the color was tested by carrying out parallel tests on diluted serum, with and without sodium sulfate in amounts equivalent to that present in the albumin analysis. The salt was found to give a decrease in color of about 1 per cent. Similarly, since the albumin filtrate contains some of the original liquid of the serum, tests were carried out to find the amount of color due to the protein-free serum filtrate. Serum samples of 2 cc. were precipitated with trichloroacetic acid. The acid of the filtrate was then neutralized and the color determined by adding alkali and phenol reagent and comparing against a standard tyrosine solution. From the color obtained it was calculated that the color due to the protein-free filtrate in the albumin aliquot amounts to about 2 per cent of the total color. It is to be seen that the diminution in color because of the sodium sulfate is about compensated by the color due to the protein-free filtrate.

Determination of Tyrosine Equivalents of Serum Proteins.—The tyrosine equivalents of the serum proteins were determined for human blood, with pooled samples of serum, by making parallel determinations by the colorimetric method and by Kjeldahl analysis. Total protein of the serum was determined on 2 cc. samples by precipitating with 5 per cent trichloroacetic acid solution, washing, and determining the nitrogen. Globulin was determined on 5 cc. serum samples by salting out with sodium sulfate and determining the nitrogen by the Kjeldahl method. The analyses were carried out in duplicate. The albumin nitrogen was obtained by subtracting the globulin from the total protein. The protein fractions were then calculated by multiplying the nitrogen by the factor 6.25 as adopted by Wu. The average value

of the factors obtained, in terms of the mg. of protein that give a color equivalent to that given by 1 mg. of tyrosine are: total protein, 16.0; albumin, 16.6; globulin, 14.4.

Calculations.—The general formula for calculating the percentage of protein from the colorimetric reading is:

$$\frac{R}{X} \times T \times \frac{100}{S} \times \frac{f}{1000} = \text{protein (in per cent)}$$

TABLE I.
*Colorimetric Analysis of Serum Proteins.**

Subject.	Diagnosis.	Albumin.	Globulin.	Ratio, albumin. globulin
		per cent	per cent	
Ne	Normal.	4 5	2 2	2.05
Co	"	5 0	2 2	2 27
Se	"	5 3	2 4	2 20
Mrs. Ga	" pregnancy.	3 8	1 9	2 00
" La	" "	4.0	2 1	1 90
Sw	Jaundice.	4.1	2 4	1 70
Fra	"	3.6	2 6	1 38
Cant	" (terminal stage).	3 4	2 9	1.17
C	" and gaseous infection.	3.8	1 7	2.20
Ho	Hyperthyroidism.	4.6	2 0	2 30
Ms	Hyperplasia of thyroid.	4 4	2 7	1 63
Sa	Carcinoma of stomach.	4 2	2 3	1 82
Du	" " tongue.	5 1	2 3	2 22
Mrs. Swa	Sarcoma	4 0	1 6	2 50
Me	Infectious arthritis.	5 3	2 3	2 30
Mrs. An	Arthritis.	5 3	2 5	2 12

* These results have been obtained in cooperation with the Department of Medicine of the University of California Medical School.

In the formula R is the point at which the standard is set, X the reading of the unknown, T is the mg. of tyrosine in the standard solution, S is the aliquot of serum used, and f the factor for the particular protein fraction being analyzed. For the albumin and globulin determinations, the colorimetric standard is set at 20 mm. and the amount of standard tyrosine solution contains 0.8 mg. The globulin represents a 0.5 cc. aliquot of serum and the albumin half of this value.

In our hands the method gave results accurate to about 5 per cent. For clinical use, it offers a rapid and relatively simple means of determining the serum proteins. A few typical values from our analytical results are given in Table I.

SUMMARY.

A colorimetric method is described for determining serum proteins based on the color developed with Folin's phenol reagent and by Howe's method of separating the proteins by sodium sulfate.

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A NOTE ON THE DETERMINATION OF THE INORGANIC PHOSPHATE OF THE SERUM ON THE FILTRATE FROM CALCIUM ANALYSIS.

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Very often the amount of blood available for analysis does not permit the determination of the number of blood constituents considered desirable if individual blood samples are to be used for each constituent. A considerable saving in blood and an increase in the number of constituents determinable could be effected if it were possible to determine several constituents on the same sample. As a step in this direction, we have developed a method for determining the inorganic phosphate on the supernatant fluid from serum calcium or ultrafiltrate calcium determinations.

EXPERIMENTAL.

The method used for the phosphate determination is that of Fiske and Subbarow (1). The chief difficulty that had to be overcome was the inhibiting effect of the excess of oxalate from the calcium determination on the color development. This was overcome by oxidizing the oxalate with potassium permanganate.

The supernatant fluid from the serum calcium determination on a 2 or 1 cc. sample can be used. The supernatant fluid from a 2 cc. sample is to be preferred as the aliquot of the 1 cc. amounts to only 0.375 of serum. The analytical procedure is as follows:

The calcium is determined by one of the common modifications of the Kramer-Tisdall method (2). With 2 cc. of serum, 2 cc. of water and 1 cc. of 4 per cent ammonium oxalate are measured accurately into the 15 cc. centrifuge tube. The supernatant fluid obtained after centrifuging is transferred to another tube to be used for the phosphate determination. A 4 cc. aliquot of this

fluid, representing 1.6 cc. of serum, is measured into an Erlenmeyer flask, 6 cc. of 10 per cent trichloroacetic acid are added to precipitate the protein, and the whole is then filtered. The first few drops of the filtrate are returned to the funnel. After the filtering is complete, 5 cc. of the filtrate, representing 0.8 cc. of serum, are measured into a 10 cc. volumetric flask, 1 cc. of 2.5 per cent ammonium molybdate in 5 N H_2SO_4 and 2 cc. of 0.1 N KMnO_4 are added. The molybdic acid is added first. The flask is warmed on a water bath until the permanganate is decolorized. The solution is then cooled to room temperature. It is very important that the solution be cooled before the sulfonic acid reagent is added, otherwise there is obtained a very intense color that is not due merely to the phosphate present. After it is cooled, 0.4 cc. of the sulfonic acid reagent are added, the volumetric flask is filled with water to the 10 cc. mark, and the contents mixed. At the same time two standards are made up, with 3 and 5 cc. of standard phosphate solution, containing respectively 0.24 and 0.4 mg. of phosphorus. The standards are made up in the usual manner described by Fiske and Subbarow (1). The colors can be compared after about 10 minutes, but should be compared again at the end of $\frac{1}{2}$ hour. The last reading is used if the color is changed. The color of the unknown after complete development was found not to change even on standing overnight if left at room temperature.¹

Calculations.—The sample used represents 0.8 cc. of serum.

¹ With a 1 cc. serum sample, 2 cc. of water and 1 cc. of oxalate solution are added and after being centrifuged and drained, 3 cc. of the supernatant fluid are taken for phosphate analysis. To the 3 cc. of supernatant fluid, 3 cc. of trichloroacetic acid solution are added and the precipitated protein filtered out. A 3 cc. aliquot of the trichloroacetic acid filtrate, representing 0.375 cc. of serum, is pipetted into a 10 cc. volumetric flask, 1 cc. of 2.5 per cent ammonium molybdate in 5 N H_2SO_4 and 2 cc. of 0.1 N KMnO_4 are added, the permanganate is decolorized, and the rest of the procedure carried out as described for the 2 cc. serum samples. A standard with 2 cc. of phosphate solution is used for comparison. Since the color obtained is only moderate, some difficulty may be experienced in matching against the standard. A useful expedient is to run in the standard solution in both cups and set them at the 20 mm. mark in the colorimeter. Then, by changing the position of the mirror, the field is darkened until differences in the settling of one division can readily be detected.

The standard contains either 0.24 or 0.4 mg. of phosphorus. Then the inorganic phosphorus of the serum, in mg. per 100 cc., is 100 times the ratio of the reading of the standard to the reading of the unknown, multiplied by the phosphorus in the standard and divided by the serum aliquot.

DISCUSSION.

In Table I are shown some of the results obtained on the supernatant fluid from calcium determinations on human serum as compared with direct determination on the original sera. The

TABLE I.

Comparison of Inorganic Phosphorus. Determinations on Serum and Supernatant Fluid from Calcium Analysis.

Specimen.	Size of sample.	Serum.	Ca supernatant fluid	Variation from serum value
	cc	mg per 100 cc	mg per 100 cc	per cent
P*	2	5.46	5.35	2.0—
K	2	2.61	2.68	2.7+
P*	2	3.31	3.28	0.9+
P*	2		3.31	0.0
P*	2	5.87	5.91	0.7+
P*	2	3.33	3.47	4.2+
J†	2	4.54	4.68	3.1+
P*	1	4.28	4.22	1.4—
P*	1	3.46	3.41	1.2—
D	1	3.11	3.20	2.9+
A	1	3.27	3.46	5.5+
Z	1	3.83	3.93	3.7+
B	1	4.50	4.45	1.1—

* Pooled sera.

† Serum from a jaundiced patient.

agreement obtained is quite good, the variation being on an average not more than a few per cent from the serum values.

We are indebted to Mrs. Thelma Starke of the University of California Hospital laboratory for suggesting the use of supernatant fluid and to Mr. J. B. Dalton for technical assistance

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COMPOSITION OF BONE.

VI. EFFECT OF MASSIVE DOSES OF IRRADIATED ERGOSTEROL.*

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Klein (1) recently reported experiments in which massive doses of irradiated ergosterol were fed to young rats. The three groups of rats all received Steenbock's Ration 2965 (2). One group served as a control. The second group received 2 gm. of cod liver oil to every 100 gm. of the ration, while the third group received massive doses of irradiated ergosterol¹ in addition to the ration.

At the end of the experimental period of 1 month the rats were autopsied and the bones were placed at our disposal for analysis. The present paper is a report of the analyses of these bones.

The serum calcium of the control rats was 10.9 mg. per cent; that of the cod liver oil group was 13.9 mg. per cent and that of the group fed irradiated ergosterol was 16.1 mg. per cent. It seemed possible that this 50 per cent increase in the serum calcium of the rats which had received irradiated ergosterol might have affected the ratio $\frac{\text{residual Ca}}{\text{P}}$ in the bones of these rats. The bones were therefore prepared and analyzed according to the method of Shear and Kramer (3). There was one notable difference in technique; phosphorus was determined by the method of Fiske and Subbarow (4) instead of by the Briggs-Bell-Doisy method. The results are given in Table I.

The mean of all the ratios obtained is 2.00 with an average deviation of ± 0.05 . This compares well with the value pre-

¹ Read at the annual meeting of the American Pediatric Society, Louis, May 20, 1929.

The irradiated ergosterol was obtained through the courtesy of Dr.rell of the Winthrop Chemical Company, Inc., New York.

TABLE I.
Bone Analyses.

Group.	Bones.	Ca	P	CO ₂	Carbonate Ca Total Ca	Residual Ca. P	Devia- tion.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Control.	Legs.	18.4	8.0	1.98	10	2.09	+0.09
		18.1	7.8	1.98			
		18.3	7.9	1.98			
	Ribs and spines.	11.2	5.4	1.06	9	2.02	+0.02
		11.6	5.3	1.01			
		11.8	5.0	1.04			
11.4		5.2					
	11.5						
Irradiated ergosterol.	Legs.	16.2	7.2	1.96	11	2.03	+0.03
		16.1	7.0	2.00			
		16.2	7.1	1.98			
	Ribs and spines.	7.1	3.2	0.85	11	2.00	0.00
		7.2	3.1	0.84			
		7.2	3.2	0.85			
Skulls.	18.0	7.7	2.57	13	2.04	+0.04	
	18.4	7.8	2.48				
	18.2	7.8	2.53				
Cod liver oil.	Legs.	15.0	6.9	1.84	11	1.91	-0.09
		14.7	6.9	1.89			
		14.9	6.9	1.87			
	Ribs and spines.	12.6	5.8	1.50	11	1.93	-0.07
		12.4	5.8	1.40			
		12.5	5.8	1.45			
Mean.....						2.00	
Average deviation.....							+0.05

viously obtained for normal rat bones by Kramer and Shear (5) in which a mean of 1.99 was found with an average deviation of ± 0.05 . For the control rats ratios of 2.09 and 2.02 were obtained;

for the rats which had received irradiated ergosterol the ratios obtained were 2.03, 2.00, and 2.04. There is thus no discernible difference between these two groups. For the cod liver oil group ratios of 1.91 and 1.93 were obtained. Although the ratios of the cod liver oil group are both lower than those of the other two groups, we do not at present attach significance to these differences because they are within the range of reproducibility of the micro technique.

SUMMARY.

1. The bones of young rats which had been fed massive doses of irradiated ergosterol for 1 month were analyzed for calcium, inorganic phosphate, and CO_2 . From these data the ratio $\frac{\text{residual Ca}}{\text{P}}$ was calculated.

2. The ratios obtained for the bones of the rats which had been fed irradiated ergosterol were the same as those obtained for the bones of control rats and of rats which had been fed cod liver oil.

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FAT EXCRETION.

VI. EXCRETION BY THIRY-VELLA FISTULAS.

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Confirming the work of Hill and Bloor (1) and Holmes and Kerr (2), Sperry (3) has recently reported the results of his research regarding lipids in the feces under varying experimental feeding conditions. Investigation as to the source of this material in the intestinal canal has been in progress in this laboratory and the following is a contribution.

In the opinion of most workers on this subject, including Sperry and Bloor (4), the presence of lipids in the feces may be due to (a) intestinal bacteria, (b) desquamated epithelial cells, (c) excretion from the blood directly or through the bile or digestive secretions, and (d) unabsorbed lipid material from the blood.

The work of Hill and Bloor (1) and Holmes and Kerr (2) indicates that the source of feces fat may not be unabsorbed food residues since there was found a large excretion of lipid on fat-free diet, appreciable fat excretion on fasting, and a lack of similarity in composition between feces fat and food fat. Intestinal bacteria and other cellular material cannot at present be evaluated, but the examination of material collected from intestinal fistulas offers a means of study of direct excretion from the blood.

Historically, this work dates back to 1864. In that year, Ludwig Thiry (5), in an effort to collect an intestinal product uncontaminated by the presence of unutilized food, made the classical Thiry fistula. He isolated a section of intestine of the desired length and region of the gut and, after again establishing the continuity of the intestinal canal, made a blind end of one extremity of his excluded loop and passed the other end through the parietal peritoneum and abdominal wall, fastening it into the skin. Collections were made by the use of a cannula.

Vella (6), a few years later, went a step farther and passed both ends of the loop of intestine through the wall, fastened them to the skin, and in this procedure was assured more perfect drainage.

In 1886, Gumilewski (7) made a low Thiry-Vella fistula and reported a continuous secretion which varied from 0.2 cc. per hour during fasting to

10.8 cc. per hour after eating. Röhmann (8), repeated the work but used longer loops and checked Gumilewski's results with low loops but found practically no secretion in high loops. Hermann (9) succeeded in making two practical loops in ten attempts and in his best loop, a high one, found it filled with 60 gm. of material in 16 days. He found that a lower loop filled much more slowly—the opposite of Röhmann's results. Ehrental (10) made an examination of the product from Hermann's loops and noted the presence of fat. He made some fistulas himself but his subjects lived only long enough for him to observe that the secretion was continuous and that microscopic examination of it revealed the presence of globules of fat. Ehrental used open loops. He believed the material was desquamated epithelial cells. Voit (11) repeated this work and found that there was from 22.6 to 36 per cent fat in the material and this comprised 0.99 to 10.05 per cent neutral fat, from 13.1 to 29.58 per cent free fatty acids, and from 3.11 to 8.23 per cent soaps. This material he attributed to intestinal secretion.

Bloor (12) collected the sample produced in a 5 day period in a Thiry fistula of 14 inches of jejunum and noted 0.72 gm. of lipid material—almost entirely fatty acids. Honigman (13) observed a patient in which the large intestine was closed off by a fistula and observed no secretion under these circumstances.

Kobert and Koch (14) in a similar case obtained about a gm. of dried material per day, containing 6.84 to 9.32 per cent fat, of which 90 per cent was free fatty acid, 9 per cent neutral fat, and 1 per cent soap, and also noted that solutions injected to kill bacteria failed to affect the fat production.

Operative Procedure.—Under ether anesthesia, a right rectus incision was made. A loop of 12 inches of ileum or jejunum, was isolated, the mesenteric vessels tied off, and the intestine divided. End to end or lateral anastomosis was carried out and the mesenteric edges were stitched in order to avoid possibility of hernia.

The skin was incised about $1\frac{1}{2}$ inches from the midline on both sides and parallel to the main incision, the slits being about $\frac{3}{4}$ inch in length. The end of the gut was brought up on each side and carried through the muscle and fasciæ obliquely in order to obtain a sphincter effect, and the serosa and muscularis were stitched to the skin edges. It was also found advisable to stitch the serous surface of the gut to the parietal peritoneum in order to prevent the gut from everting after healing.

It was found that the drawing of intestine neatly through the wall was facilitated by pushing a suitable sized tube through the incision from within out and then pushing the cut end of the gut

into the tube. The tube which contained gut was then drawn outward, thus preventing to some extent the contamination of the wall and muscles with intestinal contents. Fistulas were made at two levels—high jejunal, just below the duodenum, and low or ileal, near the cecum.

Feeding.—The dogs used in this work were a Beagle weighing 15.5 kilos and a hound weighing 20 kilos. Three diets were used: (a) Ordinary house diet. This consisted of a balanced ration of food scraps, fed once daily with water, as desired. (b) A low fat diet. Beef muscle was extracted by the continuous extraction method (4) and the dried fat-free material used as the basis of this diet. To this was added a small amount of bread and some meat

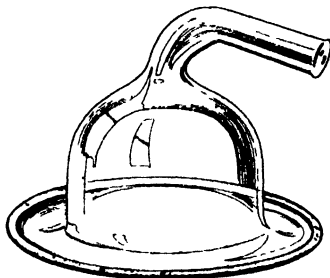


FIG. 1. Glass collector to cover fistula in accumulation of excretion. Flat surface, which is sealed to skin by use of special mixture, is sanded to improve adhesion. The edge of this surface is rolled to prevent cutting the skin. Exit tube to which balloon is tied has rolled end to prevent tie from slipping. Figure is about two-thirds the size of collectors used to best advantage and which measure 6 cm. in diameter across the base.

extract (fat-free) for flavoring. Although this diet was not entirely fat-free it was a very low fat diet, and in comparison with the ordinary diet relatively fat-free. (c) A high fat diet. For this ration, beef suet was added in moderate amounts to the ordinary diet. The animals were given the diets for 3 to 5 days before collections were begun.

Collections.—In order to avoid irritation of the mucous surfaces and as a result an abnormal secretion, insertion cannulas were not used but special glass collectors were made which enclosed the openings of the loops as shown in Fig. 1.

The cannulas were held in place with a cement made of rosin, castor oil, and beeswax in proportions determined by the adhesive power of the product when tested. A useful mixture contained rosin 30 gm., castor oil 2 cc., beeswax 21 gm. In preparing the cement the rosin was melted in a glass container, the wax then added, and the oil dropped in a drop at a time until proper adhesive power and consistency was attained. The adhesion of glass to the hand was used as a test in producing the correct mixture. Complete 24 hour samples were collected. Both high and low loop samples consisted of a thin, milky fluid of disagreeable but not foul odor. The odor may be described as like butyric acid. The high loop samples contained more of a suspended soapy-looking material than did the lower. The low loop samples were of lesser density, and the suspended particles were smaller, but settled more readily to the bottom of the vessel.

It was noted that the excretion was practically absent or greatly diminished in cases of diarrhea, natural or provoked. Collection of the complete sample was difficult and there were many failures owing to leakage due to movements of the animal.

Analytical Procedure.

Fraction 1.—The sample collected was acidified and well shaken in a separatory funnel with about 30 cc. of ether, then allowed to stand overnight to get rid of the emulsion. The layers were then separated and the watery liquid washed three times with ether. The combined ether extract, usually tinged with yellowish pigment, was washed once with distilled water, the ether evaporated, the residue taken up with petroleum ether, the extract filtered, the solvent evaporated, and the residue dried to a constant weight.

Fraction 2.—The watery residue was then treated with NaOH (to make about 20 per cent solution) on the steam bath for at least 4 hours, then acidified and extracted and the residue purified, dried, and weighed, as in Fraction 1.

Fraction 3.—The dark colored residue from Fraction 2, insoluble in petroleum ether, was dissolved in ether, filtered, evaporated to dryness, taken up again with ether; then the ether was removed by evaporation and the residue dried and weighed. In spite of its insolubility in petroleum ether this substance was obviously fatty and so is included in the results.

A great many experiments were run on these two animals, and Tables I and II contain the results of typical successful experiments.

TABLE I.
Fatty Material Excreted in High Fistula (Beagle Dog).

Date.	Excretion.	Fatty material in:			
		Fraction 1.	Fraction 2.	Fraction 3.	Total.
Normal diet.					
1927	cc.	gm.	gm	gm.	gm
Oct. 22	82	0.011	0.006	0.0202	0 037
" 27	76	0.011	0.005	0.0186	0 035
Nov. 1	64	0.009	0.006	0 0210	0 036
" 8	68	0.010	0.005	0.0201	0 035
" 12	69	0.008	0.005	0.0220	0 035
" 16	80	0.016	0.004	0.0200	0 040
Average...	73	0.011	0.005	0 020	0.036
High fat diet.					
1928					
Feb. 2	61	0.010	0.006	0 020	0.036
" 10	44	0.009	0.005	0.018	0.031
" 11	70	0.014	0 005	0.016	0.035
" 16	46	0.012	0.005	0.009	0.026
Mar. 4	50	0.011	0.005	0.014	0.030
" 8	38	0.006	0.005	0.018	0.023
Average...	51	0.010	0.005	0.016	0.030
Low fat diet.					
1927					
Nov. 23	51	0.006	0.006	0.018	0.030
" 25	64	0.016	0.007	0.016	0.039
" 28	81	0.024	0.004	0.021	0.049
Dec. 1	78	0.016	0.004	0.024	0.044
" 2	48	0.011	0.005	0.018	0.034
" 5	36	0.004	0.005	0.014	0.023
Average...	60	0.013	0.005	0.018	0.037

DISCUSSION AND SUMMARY.

The results indicate that a considerable amount of fatty material was formed and excreted through these loops of intestine. The

amount excreted seemed to be independent of diet since it was much the same for both loops whether the diet was high, low, or

TABLE II.
Fatty Material Excreted in Low Fistula (Hound).

Date.	Excretion.	Fatty material in:			
		Fraction 1.	Fraction 2.	Fraction 3.	Total.
Normal diet.					
<i>1927</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Dec. 8	48	0.017	0.009	0.032	0.058
" 10	50	0.018	0.014	0.030	0.062
" 14	40	0.014	0.008	0.026	0.048
" 16	41	0.014	0.007	0.028	0.049
" 18	47	0.016	0.009	0.029	0.054
" 22	30	0.011	0.008	0.021	0.040
Average...	42.7	0.015	0.009	0.028	0.052
High fat diet.					
Dec. 28	62	0.020	0.016	0.020	0.056
" 30	50	0.017	0.014	0.027	0.058
<i>1928</i>					
Jan. 3	46	0.014	0.008	0.028	0.050
" 5	32	0.010	0.006	0.023	0.039
" 8	60	0.018	0.009	0.024	0.051
Average...	50	0.013	0.009	0.020	0.042
Low fat diet.					
Jan. 11	56	0.014	0.009	0.027	0.050
" 14	48	0.012	0.008	0.026	0.046
" 18	36	0.011	0.009	0.028	0.048
" 22	51	0.014	0.010	0.027	0.051
" 24	48	0.013	0.007	0.017	0.037
" 27	60	0.018	0.010	0.022	0.050
Average...	50	0.014	0.009	0.025	0.047

average in fat content. The amount excreted when calculated on the basis of body weight was about the same for both loops, 2.2 mg. per kilo per day for the high and 2.35 for the low, indicating

that the level of the intestine had in these cases no definite influence.

Of the fatty material excreted about one-half was insoluble in petroleum ether but soluble in ether. Of the petroleum ether-soluble material, most of it—about two-thirds—was directly extractable from the acidified secretion, therefore not in any sort of combination. The remaining third required treatment with strong alkali to render it extractable and may, therefore, have been contained in cellular material, which is also true of Fraction 3.

It has been found that the operative procedure is carried out as described, the loops will remain open an indefinite period and continue in position and actively secretory for a period of 3 years. Further examination of the secretion from these loops after such a period is indicated to determine whether or not the secretion varies in content from loops recently operated upon.

The author is indebted to Dr. W. R. Bloor of the Department of Biochemistry for his continued interest and advice throughout the work.

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THE EFFECT OF HYDROGEN ION CONCENTRATION ON SAPONIN HEMOLYSIS.

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The purpose of the present study has been to determine the change in the resistance of corpuscles to saponin with changes in pH, the acids employed being the common inorganic acids. Ponder (1) has shown that the rate of saponin hemolysis is increased in the presence of certain acids and has applied the equation $C_1 = R (C_2 + k_R)$ to systems in which an acid is the accelerating agent.

Details of technique in preparing the red blood cell suspensions and measuring the rate of hemolysis will be found elsewhere (2, 3), but to explain the significance of the terms in the equation given above, it is necessary to refer to certain features of the procedure which Ponder has adopted in his studies of hemolysis and which have been followed in a portion of this investigation. In determining the effect of an agent which either accelerates or retards the action of saponin, a time-dilution curve is first constructed which shows the relation of the time taken for complete hemolysis of a standard cell suspension to the dilution of saponin, in the absence of inhibiting or accelerating agents, the determinations being made at a constant temperature. The dilutions of saponin are usually 1 part in 10,000, 20,000, 30,000, etc. The concentrations corresponding to these dilutions (δ_1) are expressed in the above equation by C_1 , the value of which in these experiments is the number of mg. of saponin contained in the tube to which the standard red blood cell suspension is added.

Upon introducing into the simple cell-saponin system an agent which hastens hemolysis, a different time-dilution curve is obtained. For a given dilution of saponin complete hemolysis of a

standard cell suspension now occurs in a shorter time. Accordingly, by referring to the original time-dilution curve, the dilution of lysin (δ_2) may be determined, which by acting alone would

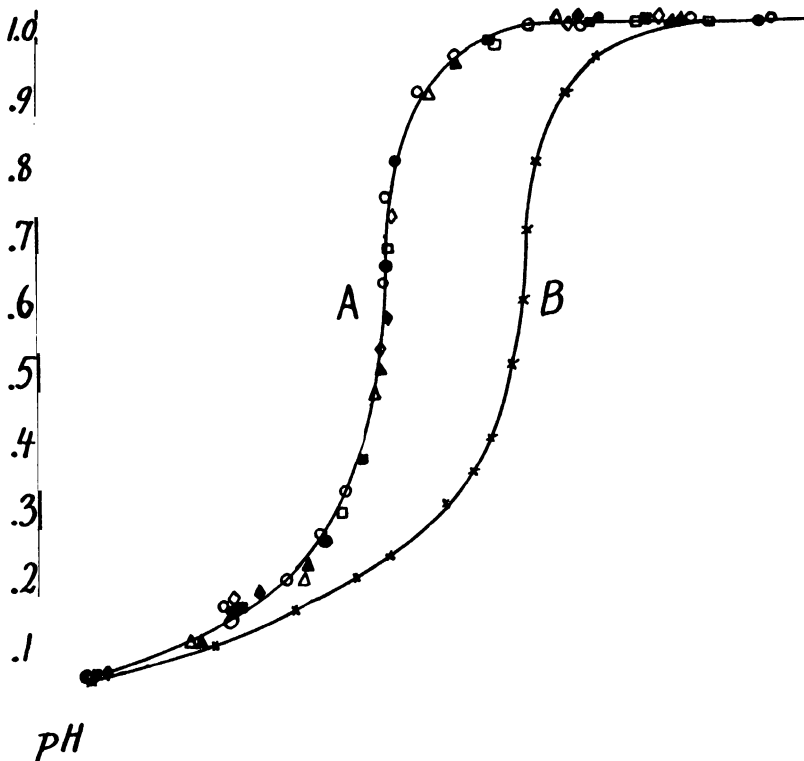


FIG. 1. Curve A represents the relation of the resistance, R , of the red blood corpuscle to the initial pH of the saponin solution. Dilution of saponin, $\delta = 30,000$. The clear circles, squares, triangles, and rhombs represent the data for dog corpuscles, with hydrochloric, nitric, sulfuric, and phosphoric acids, respectively. The solid circles, squares, etc., represent the data for human corpuscles, with the same acids. Curve B represents the relation of the resistance of the corpuscles to the pH of the cell-saponin-acid system when hemolysis is complete.

have produced complete hemolysis in the same time taken in the presence of the accelerating substance. The concentration corresponding to this dilution is C_2 . If the values for C_1 are plotted against the corresponding values for C_2 , a straight line is

obtained which makes an intercept on the C_2 axis. This intercept is represented by k_R . If the straight line passes through the origin, $k_R = 0$ and $R = \frac{C_1}{C_2}$. Ordinarily, the value of k_R is small, being treated by Ponder as an empirical constant, probably an expression of the fact that the real relation between C_1 and C_2 is not linear, but given by a flat curve.

R is the resistance constant of corpuscles by means of which the effects of two or more hemolytic systems may be compared. For example, referring to Curve A in Fig. 1, it will be seen that corpuscles suspended in a solution of saponin in saline ($\delta = 30,000$), acidified with an inorganic acid to an initial reaction of $\text{pH} = 3.0$ have 0.25 the resistance which they exhibit at a pH of about 4.5, or in the control experiment, in which saponin is dissolved in neutral saline.

Curve A, in Fig. 1, represents the data obtained with human and dog corpuscles, in each case the four inorganic acids, hydrochloric, nitric, sulfuric, and phosphoric, being employed, the dilution of saponin (Merck) being 30,000. Complete studies have also been made with $\delta = 10,000$ and 20,000. With the dilution of 10,000, in the presence of acids, hemolysis may be so rapid as to introduce a significant experimental error. More consistent results are obtained with $\delta = 20,000$, and these have been found to approximate closely the data obtained with $\delta = 30,000$, as may be seen by examining the curves in Fig. 5. When the saponin concentration is relatively low, $\delta > 40,000$, the end-point of complete lysis is not very sharp and this may likewise introduce an appreciable experimental error. Accordingly, while valuable data may be obtained over a wide range of dilutions of saponin, the most reliable results, with the methods employed in the present work, are observed with $\delta = 20,000$ and $\delta = 30,000$. Dog and human corpuscles have about the same resistance to saponin over the range of hydrogen ion concentrations represented by Curve A in Fig. 1.

This curve, which is based upon the pH values at the beginning of hemolysis, shows that the acceleration of saponin hemolysis depends upon the hydrogen ion concentration and is approximately the same for the four inorganic acids studied. The shape of the curve, moreover, suggests a resemblance to a titration curve.

It is obvious, however, that any relationship that may exist between the hydrogen ion concentration and the resistance of corpuscles is likely to be a relationship involving, not the initial hydrogen ion concentration, but some concentration intermediate between the initial concentration and that at the end of hemolysis, since, as has been previously shown (3), acids, even in very dilute solutions, react rapidly with the constituents of the red blood corpuscle. In the more concentrated solutions, the neutralization of the acid produces relatively little effect on the hydrogen ion concentration, whereas in the dilute solutions, the neutralizing effect of the cell buffers causes much greater shifts in pH.

The time required for complete hemolysis being determined by Ponder's method (2), parallel determinations were made in which neutralization of the acid in the saponin solutions was measured by means of a quinhydrone electrode. In each of these determinations, 4 cc. of the red blood cell suspension were added to 16 cc. of the acid-saponin solution. Quinhydrone did not seem to affect appreciably the rate of saponin hemolysis and it was assumed that the hemoglobin liberated from the 0.1 cc. of corpuscles, contained in the 4 cc. of suspension, did not influence the determinations materially because of the effect of the hemoglobin in altering the ratio of oxidant to reductant. Readings of the E. M. F. were taken before adding the cells and at intervals of 10 to 30 seconds during hemolysis, until equilibrium was attained. From the results obtained in this way, it was possible to determine the pH shift from the initial value which occurred in the interval required for complete hemolysis, as determined in the parallel determinations by Ponder's method. This shift may be represented by ΔpH . The value of $\text{pH} + \Delta\text{pH}$, in any given case, is therefore the pH at the end of hemolysis.

Curve B, Fig. 1, is based on the values of $\text{pH} + \Delta\text{pH}$. The actual relation between pH and the resistance of cells to saponin would probably be represented by a curve plotted between Curves A and B. Such a curve, except for the portion representing the lower values of R , would also bear some resemblance to a titration curve. (Compare, for example, these curves with the titration curves for various proteins given by Cohn (4).)

The value of R , at any given pH, being approximately proportional to the amount of lysin needed to produce a given effect, as

compared with the amount needed in the absence of acid, the preceding results indicate that the acceleration of saponin hemolysis by acids may depend either (a) on the formation of a dissociable acid-saponin compound, or (b) on a similar combination of hydrogen ion with one or more constituents of the red blood corpuscle.

Experiments with "Acid" and "Basic" Saponins.—With few exceptions, the saponins are either neutral or weakly acid. The product used in this work, Merck's pure saponin, was slightly acid. In a dilution of 1:30,000, in saline, the pH was 6.00; in a solution containing 1 part in 2500, the pH was 5.76. Saponin reacts both with acids and bases, and accordingly, small amounts of "acid" and "basic" saponins were prepared. 2 gm. quantities of saponin were dissolved in 50 cc. of 0.1 N HCl and, after being cooled in the refrigerator, the acid-saponin was precipitated with acetone. The precipitate was redissolved in a small volume of warm alcohol, reprecipitated with acetone, filtered, and dried. Alkali-saponin was prepared by dissolving 2 gm. portions of saponin in 50 cc. of 0.1 N NaOH, cooling, and precipitating with alcohol or, preferably, an alcohol-ether mixture. The precipitate was redissolved in water and reprecipitated with alcohol-ether, filtered, and dried. Portions of the commercial saponin were further purified by being dissolved in water and precipitated with alcohol-ether and alcohol-ether-acetone mixtures.

The preparation of "acid" and "basic" saponins did not result in the hydrolysis of any of the saponin, as shown by the absence of reducing sugars at any stage in the preparation and in the final products.

The acid-saponin, dissolved in physiological salt solution, gave the following values: $\delta = 2,500$, pH 4.50; $\delta = 5,000$, pH 5.42; $\delta = 10,000$, pH 5.85; $\delta = 20,000$, pH 5.96. The alkali-saponin, dissolved in physiological salt solution gave the following values; $\delta = 2,500$, pH 7.28; $\delta = 5,000$, pH 6.99; $\delta = 10,000$, pH 6.77; $\delta = 20,000$, pH 6.64. The original saponin, similarly dissolved, gave the values: $\delta = 2,500$, pH 5.76; $\delta = 5,000$, pH 5.89; $\delta = 10,000$, pH 5.91; $\delta = 20,000$, pH 5.96; $\delta = 30,000$, pH 6.00. The repurified saponin was somewhat less acid, giving the following values: $\delta = 2,500$, pH 6.05; $\delta = 5,000$, pH 6.10; $\delta = 10,000$, pH 6.13; $\delta = 20,000$, pH 6.17; $\delta = 30,000$, pH 6.24.

The results of the experiments with the "acid" and "basic"

saponins are represented by the curves in Fig. 2. These show that the formation of a dissociable acid-saponin compound is not the cause of the acceleration of saponin hemolysis by acids. In fact, acid-saponin was much less effective as a hemolytic agent than the original saponin. In a dilution of 20,000, hemolysis occurred in 1 minute when the original saponin was used, but, with acid-saponin, hemolysis was not complete even at the end of 30 minutes. Alkali-saponin was likewise less hemolytic than either the untreated or repurified preparations.

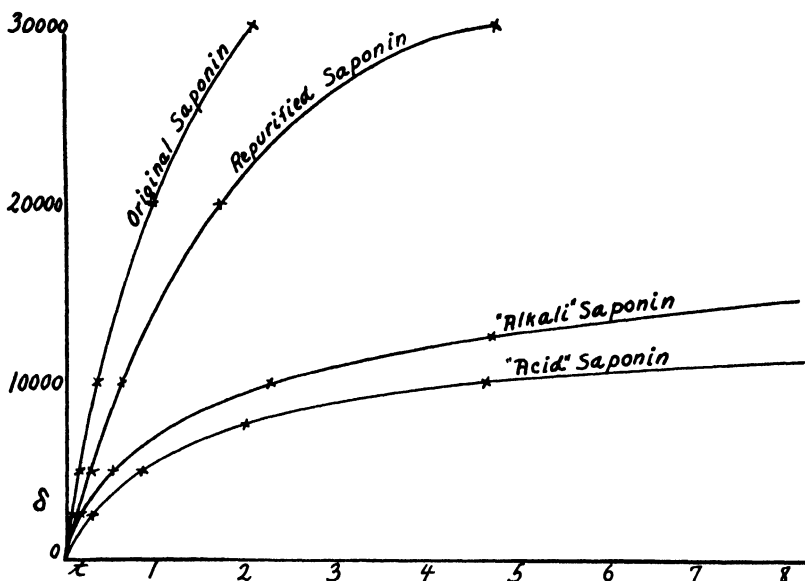


FIG. 2. Time-dilution curves of various saponin preparations.

Experiments with Red Blood Corpuscles, Treated with Acid and Alkali.—When erythrocytes are treated with dilute acid, their resistance to hemolysis by saponin is diminished. Ponder (1) has observed this effect in the case of glutamic and aspartic acids. On the other hand, when cells are treated with arginine, which inhibits hemolysis, their resistance to saponin increases.

Dog corpuscles, centrifuged from 50 cc. of a standard suspension, were treated with 50 cc. of 0.001 N HCl (the HCl was made up in salt solution, the concentration of the salt being adjusted so that

the acid solution would be isotonic with the cells). After 5 minutes, the acid-cell suspension was centrifuged; the cells were again

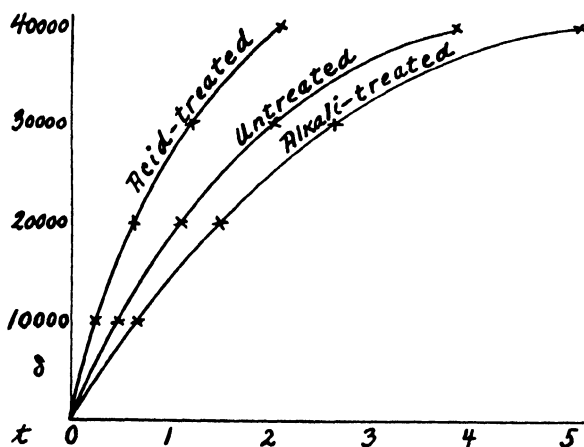


FIG. 3. Time-dilution curves showing the effect of previously treating the red blood cells with acid and alkali.

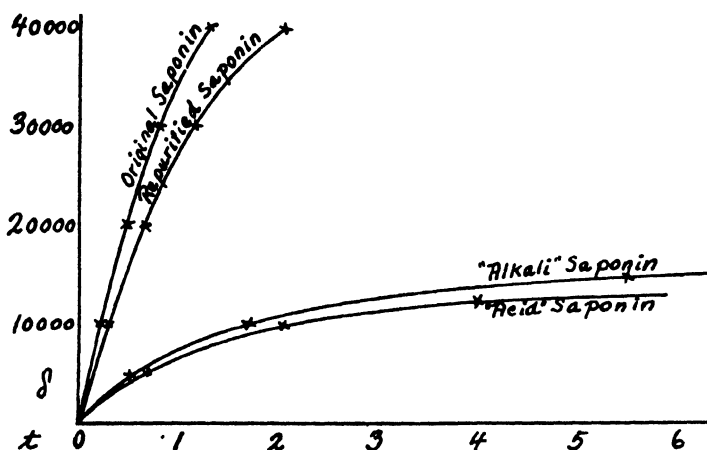


FIG. 4. Time-dilution curves showing the effect of various saponin preparations on red blood cells, previously treated with acid.

suspended in 50 cc. of acid saline, centrifuged and washed three times with physiological salt solution. Another 50 cc. portion was

similarly treated with 0.001 *N* NaOH in saline, the cells being suspended in the alkaline saline but once, in view of the rapid hemolysis which occurs on repeated treatment with alkali.

The behavior of saponin toward the acid- and alkali-treated cells is shown by the curves in Fig. 3. From these it is clear that

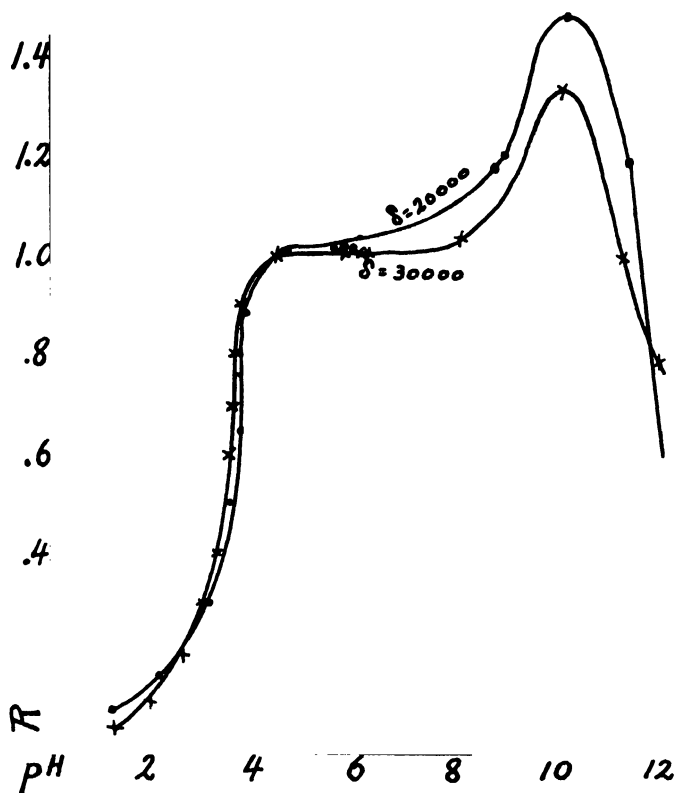


FIG. 5. The relation of the resistance, R , to the initial pH of saponin solutions. Dilutions, $\delta = 20,000$ and $\delta = 30,000$.

the effect of the acid in accelerating hemolysis and of alkali in inhibiting it must depend largely on the action of these on the corpuscle. At the beginning of hemolysis, the saponin solution, $\delta = 30,000$ had a pH value of 5.96 in all cases. On addition of the washed, untreated cells, the pH shifted to an equilibrium value of 7.01 when hemolysis was complete, the shift being due to the

liberation of the cell buffers. On the other hand, the acid-treated cells caused a shift in the opposite direction, from pH 5.96 to 5.88. As compared with these observations were those noted with the alkali-treated cells which produced a shift from pH 5.96 to pH 7.40.

A comparison was also made of the action of "acid," "basic," and approximately neutral saponins on the acid-treated cells. The results, represented by the curves in Fig. 4, show that each of the saponin preparations hemolyzed the acid-treated cells more readily than the untreated cells (compare with Fig. 2). Here, also, the "acid" and "basic" saponins were much less hemolytic than either the untreated or repurified preparations. For example, in a dilution of saponin, $\delta = 20,000$, hemolysis of the acid-treated cells, with the original saponin, occurred in 0.52 minute, whereas with the "basic" saponin, hemolysis occurred in 13 minutes, and with the "acid" saponin, 17 minutes were required.

Near the neutral point, the resistance of the red blood corpuscles increases slightly with increasing pH, to approximately pH 8.5, after which the resistance increases more rapidly, reaching a maximum at about pH 10. The effect of further increase of alkalinity on saponin hemolysis cannot be studied satisfactorily, owing to the independent hemolytic effect of the alkali. In the experiments upon which the curves in Fig. 5 are based, the saponin solutions were adjusted to a given pH by the addition of either HCl or NaOH, the pH being determined colorimetrically.

DISCUSSION.

The effect of hydrogen ion concentration on saponin hemolysis is apparently twofold. The reaction between acid and saponin yields an ionizable acid-saponin compound which retards hemolysis, an effect which manifests itself over the entire range of acidity and which probably accounts for the higher resistance of corpuscles observed in a saponin solution, acidified to pH 4.5, or higher, than is noted in a saponin solution prepared in neutral saline. The second and predominant effect, and the one responsible for the acceleration of hemolysis, is due to the reaction of the acid with the cell constituents.

Coulter (5), in studying the cataphoresis of red blood cells, determined that their movement in an electric field was a function

of the hydrogen ion concentration. For normal sheep corpuscles he obtained the value pH 4.6 for the isoelectric point, that is, the point at which no movement of the cells occurred. On the alkaline side of the isoelectric point the cells are negatively charged, the charge increasing with the alkalinity, whereas on the acid side, the charge is positive and increases with the acidity. The behavior of corpuscles toward acids and bases corresponds with that found for protein. On the acid side of the isoelectric point, the red blood corpuscles combine chemically with H^+ and Cl^- ions, whereas on the alkaline side, combination occurs with cations.

A variety of phenomena, such as agglutination and stability of blood cell suspensions, seem to be linked in some way to the electric charge of the red corpuscles. Coulter (5) has observed that the optimum for agglutination of normal cells is at pH 4.75, a point at which "the cells exist most nearly pure, or least combined with anion and cation." From their studies of the influence of electrolytes on the stability of red blood corpuscle suspensions, Oliver and Barnard (6) are led to conclude that a red blood cell when suspended in fluid reacts in regard to its electrokinetic properties as if it were possessed of a surface of globulin.

Judging from the curves in Figs. 1 and 5, the behavior of saponin is likewise modified by the electric charge of the corpuscles. The greater the positive charge, the less the resistance to saponin, the point of inflection of the curves appearing approximately at the isoelectric point of the corpuscles. On the alkaline side of the isoelectric point, as the cells become negatively charged, their resistance to saponin increases somewhat, but not markedly so until after a pH of about 8.5 is attained. The second inflection in the curve (Fig. 5) occurs approximately at the point which marks the reversal of permeability of the corpuscle to anions, as has been recently shown by Mond (7). This investigator observed that erythrocytes treated with alkali become impermeable to anions but permeable to cations, the reversal occurring between pH 8.0 to 8.3. This value corresponds closely to the isoelectric point of globin (from the erythrocytes of cattle), determined by Osato (8) to be pH 8.1 ± 0.1 . Accordingly, Mond concludes that the membrane of the red blood corpuscle contains protein phases consisting of globin. While the present writer is inclined to accept the view of Mond with regard to the possible importance of this protein in red

blood cell permeability, he is unaware of any direct evidence that globin, as such, is a constituent of the red blood corpuscle.

SUMMARY.

The relationship of pH to the resistance of dog and human erythrocytes to saponin has been studied and the results represented by means of curves.

The effect of inorganic acids and bases on saponin hemolysis is believed to depend principally on their chemical combination with globulin and possibly other proteins of the cell membrane, such as globin.

On the acid side of the isoelectric point of the red blood corpuscle (pH about 4.6), as the positive charge increases, the resistance of the cell to saponin diminishes. On the alkaline side of the isoelectric point, as the cells become more and more negatively charged, their resistance to saponin increases gradually to about pH 8.5, after which the resistance increases more rapidly, reaching a maximum at about pH 10.

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THE EFFECT OF DRYING AND OF SULFUR DIOXIDE UPON THE ANTISCORBUTIC PROPERTY OF FRUITS.*

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The most important antiscorbutic foods are now known to be the fruits and vegetables, and it is usually considered that these foods are chiefly, if not only, valuable for this property when used fresh. Holst and Fröhlich (1) showed that cooking and canning or drying greatly decreased the antiscorbutic value of a variety of foods. Since the danger of scurvy is greatest under those circumstances under which an adequate supply of fresh foods is apt to be difficult to obtain, much interest has always attached to the effect of various methods of preservation upon the vitamin C content of foods. Numerous studies have been made upon the citrus fruit juices, cabbage, milk, potatoes, tomatoes, and a few other fruits and vegetables. In general as indicated by the work of Harden and Zilva (2), Hess and Unger (3), Givens and McClugage (4), and Goss (5), a large proportion of the vitamin is preserved in citrus products dehydrated by suitable means, particularly *in vacuo* and at relatively low temperatures. Most of these studies have been made upon laboratory products, but a few have dealt with commercial specimens (Goss (5) and Humphrey (6)).

A number of tests of dried cabbage has been made by Holst and Fröhlich (1), Cohen and Mendel (7), Delf and Skelton (8), and Ellis, Steenbock, and Hart (9), with varying results, the vitamin being more or less decreased even when the cabbage was dried in carbon dioxide, if the temperature exceeded 65°. The work of

* Published with the consent of the Director of the California Agricultural Experiment Station. This investigation was suggested by A. W. Christie formerly in charge of fruit drying investigations at this station.

Hess and Unger (3) with dried carrots and dried prunes indicated also quite complete loss of the antiscorbutic property during drying, although detail of method of drying was not described. Disappointing results were reported by Givens and McClugage (4) with dried potatoes, and by Chick, Hume, and Skelton (10), and Shorten and Ray (11), with various dried fruits and vegetables. The latter investigators report that tomatoes retain a good deal of the vitamin after being sun-dried, potatoes and cabbage some, but that carrots, spinach, turnips, and various leaves, lose all.

Apparently the more acid foods, such as the citrus fruits and tomatoes, are not only more richly endowed than others with the antiscorbutic vitamin, but also are better able to resist destruction by drying or processing. Alkalinization was shown by Hess and Unger (12) and by La Mer, Campbell, and Sherman (13) to increase the rate of loss. The influence of oxygen has been shown also to be deleterious by a number of workers, but neither acidity nor oxidation produce destructive effects which are consistent. Kohman, Eddy, and Carlsson (14), in an interesting series of experiments have shown that the exhausting of apples, spinach, and peas during commercial canning has a protective effect upon this vitamin, probably because of delayed oxidation. But a complete preliminary exhaust or removal of oxygen by respiration furnished no increase in protection of the vitamin in the case of peaches (15).

Because of these inconsistencies, and because of the economic importance of the commonly used dried fruits, this investigation into the effect of known conditions of drying upon the antiscorbutic property of certain fruits was undertaken. No previous study of commercially produced dried fruits was available with the exception of one by Eckman (16), in which peaches, prunes, apricots, apples, cherries, and berries were tested, but the results were reported without statement as to mode of drying or comparison with the corresponding fresh fruits. Negative results were obtained except in the case of the dry peaches, which were found to be partly protective. Peaches only were used in the experiments here reported, although we have data upon prunes and apricots which substantiate our conclusions, and which will be published later.

Preparation of Fruit.

The fruit was prepared by members of the staff of the Fruit Products Laboratory of the College of Agriculture of the University of California. Fresh, completely ripe peaches of the Muir variety, grown near Walnut Creek, California, were picked, pitted, ground in a food chopper, and at once packed in small tin contain-

TABLE I.
Preparation and Composition of Fruit.

Fruit.	Lot.	Method of preparation.	Moisture.	Net shrinkage		Sulfur dioxide.
				Fresh weight	Dried weight	
			per cent			parts per million
Peaches, Muir variety.	Fresh.	Cut, pitted, ground, sealed cold in 8 oz tin containers, frozen and kept at -17° .	79 57	1 00		
" "	Sun-dried, unsulfured	Cut, pitted, sun-dried 8 days, dried in stack 6 days.	19 70	3 93		
" "	Sun-dried, sulfured	Cut, pitted, sulfured overnight, sun-dried 8 days, dried in stack 6 days	15 40	4 14		1875
" "	Dehydrated, unsulfured	Cut, pitted, dried in dehydrator at 63° for 20 to 24 hrs.	19 12	3 96		
" "	Dehydrated, sulfured.	Cut, pitted, sulfured overnight, dried in dehydrator at 63° for 20 to 24 hrs.	16.01	4 11		1840

ers, which were sealed without heating, and frozen hard. The fresh fruit was kept in a freezing room at approximately -17° until it was ready to be fed. There seemed to be no change in vitamin content throughout several months storage when fresh fruit was kept under these conditions. Other portions of Muir peaches from the same trees were dried in various ways, as indicated in Table I. None of the fruit was peeled. After being

dried all peaches were ground in a food chopper, packed without heating in 8 oz. cans, and stored at approximately -17° .

The moisture content of all preparations was determined by drying at 70° *in vacuo* to constant weight, and sulfur dioxide was determined by distillation into standard iodine solution, according to the official method of the Association of Official Agricultural Chemists (17).¹ These results are shown in Table I. The moisture content of these products is less than that usually found in marketed fruit, since these figures represent dry yard fruit, which was not subjected to the usual steam or water processing which the packers carry out.

Method of Feeding.

The basal diet used for all the feeding tests was a slight modification of that proposed by Sherman, La Mer, and Campbell (18).

Basal Diet; Scurvy-Producing.

Rolled oats.....	69	Cod liver oil, per day, per
Skim milk powder (baked at		animal fed separately....
110° for 2 hours).....	30	2 cc.
Sodium chloride.....	1	

On this diet, supplemented daily by 12 cc. of canned tomato juice, young guinea pigs grew rapidly and remained free from scurvy. Without the tomato or other source of vitamin C, the animals succumbed with severe scurvy in 25 to 35 days. All guinea pigs, weighing about 300 gm. at the beginning, were kept in separate metal cages on shavings, with fresh water and basal diet before them at all times. They were weighed twice a week, and were kept on the protective dosage of fruit for 90 days. All that died or that were sacrificed at the end of the test period were examined for signs of scurvy. Hemorrhagic areas in intestines, peritoneum, joints, and gums were examined, and fragility of bones, looseness of teeth, and beading of ribs were noted.

The test doses of fruit were given daily separately from the rest of the diet, and full consumption of these doses was insured. In

¹ We acknowledge with thanks the assistance of Emil M. Mrak, Wong Yu Fong, and H. M. Reed who prepared the fruit and made the moisture and sulfur dioxide determinations under the supervision of Professor W. V. Cruess and P. F. Nichols of the Fruit Products Laboratory.

Table II are summarized the results of the feeding tests with the peach preparations. The marked protective action of the sulfured fruit of both dehydrated and sun-dried varieties appears to indicate complete retention of the antiscorbutic property of the fresh peach. There would appear to be slightly better retention in the

TABLE II.
Relative Antiscorbutic Value of Fresh and Dried Peach Products.

Fruit.	Amount fed daily.		No. of animals.	Average body weight.				Length of period.	Remarks.
	gm.	Equivalent in fresh fruit.		Initial.	Maximum.	Final.	Gain or loss per wk.		
	gm.	gm.		gm.	gm.	gm.	gm.	days	
Fresh peach (Muir).	5	5.0	2	416	542	329	-6	90	Mild scurvy.
	8	8.0	4	333	513	434	8	90	No " "
	10	10.0	3	320	573	570	19	90	" "
Dehydrated peach, un- sulfured.	1	3.9	2	363	386	242	-30	30	Severe scurvy, died.
	2	7.9	2	316	346	229	-14	45	" " "
	5	19.8	2	328	408	250	-11	46	" " "
	10	39.6	2	367	409	217	-30	32	" " "
Dehydrated peach, sul- fured.	1	4.1	3	345	508	499	12	90	No scurvy.
	2	8.2	3	319	484	479	13	90	" "
Sun-dried peach, un- sulfured.	1	3.9	2	306	369	224	-14	42	Severe scurvy, died.
	2	7.8	2	319	365	216	-21	38	" " "
	5	19.6	3	315	583	219	-16	41	" " "
	10	39.3	2	304	404	215	-27	33	" " "
Sun-dried peach, sul- fured.	1	4.2	5	340	478	413	6	90	1 animal, mild scurvy,
	2	8.3	3	326	491	473	11	90	others, no scurvy. No scurvy.

dehydrated than in the sun-dried product, a result which is not unexpected in view of the probably greater opportunity for oxidation in the latter case. All animals fed the unsulfured fruit, both sun-dried and dehydrated, succumbed nearly as early as did those on basal diet alone. It proved impossible to feed larger doses than 10 gm. of these products. There was little variation

in the response of the animals to the fruit feeding, and quite uniform conditions were discovered on autopsy. The only abnormality observed in the animals fed the sulfured fruit was a slight chalkiness and brittleness of the incisors. These are now being examined chemically and microscopically.

Similar but less pronounced effects are now being obtained in tests upon prunes and apricots. The effect of lye-dipping upon the preservation of vitamin C in both sulfured and unsulfured prunes remains to be discovered, and experiments upon this, as well as upon sun-dried and dehydrated sulfured prunes, and upon apricots sulfured lightly and heavily, are now under way.

The minimum protective dose of fresh peaches, 8 gm., is slightly greater than that mentioned by Kohman, Eddy, Carlsson, and Halliday (15), but since they do not give the variety of peach used, because they used random specimens from the market, a close comparison is scarcely to be expected. It is probable that Hess and Unger (3) and Eckman (16) used unsulfured prunes in their negative experiments, although there is a possibility that Eckman used sulfured peaches in the experiments in which peaches showed some protective effect.

A few tests of the hydrogen ion concentration of these fruit preparations were made by Mr. H. M. Reed of the Fruit Products Laboratory. He used both electrometric and colorimetric methods, but was able to obtain satisfactory results chiefly with the hydrogen electrode. The technique of this determination is now being studied and more satisfactory figures for the pH of the later 1928 fruit samples will undoubtedly be available. The sun-dried sulfured peaches gave 4.2 as compared with the usual 4.0 to 4.4 of fresh peaches. There would appear from these limited observations to be no necessary relation between the sulfuring and the pH of these fruits.

The mechanism of protection of vitamin C by the sulfur dioxide treatment in these fruits is of interest and is being studied in this laboratory. Whether the result is due to decreased oxidation because of a surface coagulation of the fruits, or because of the reducing action of the sulfur dioxide, or to greater stability of the vitamin in the possibly more acid medium provided by the sulfurous acid, or to some other cause remains to be discovered. In any case, a definite advantage in an important phase of dried

fruit nutritive value, appears to be derived from the sulfur dioxide treatment during drying.

In view of the long disputed question as to the possibly deleterious effects of sulfurous acid in dried fruits, these findings are somewhat surprising. Food Inspection Decision No. 76 of the United States Department of Agriculture which is widely quoted in textbooks, states that the total amount of sulfur dioxide in the finished product must not exceed 350 mg. per kilo. This decision is not enforced because of the confusion of opinions and the lack of convincing data as to the actual effects of sulfuring upon the economic and physiological aspects of the use of such dried fruit. Much of the sulfured dried fruit marketed in this country carries more than 1000 parts of sulfur dioxide per million. Certain European states, France, for instance, have recently begun to enforce a minimum of 1000 parts per million. In Hungary, Germany, and Czecho-Slovakia, 1250 parts per million, in England 2000, and in Canada 2500 parts per million are the legal minima. New York has named 2000 parts per million as minimum, and New Hampshire has recently prohibited sulfur dioxide in any amount whatever. Most of the states, however, have accepted the federal ruling indicated in Food Inspection Decision 89, issued in 1908, that "ordinary" quantities of sulfur dioxide if stated on the label are not objectionable. The objection to excessive sulfuring appears now to be rather on the score of the possible marketing of excessively watery dried fruits rather than based on the danger of physiological injury resulting from their ingestion.

It seems desirable therefore to know at what level of sulfur dioxide content the protective action upon vitamin C is effective, in order that legal minima, if these are to be established, may be made most advantageous in the nutritive sense. Further experiments upon prunes and apricots with widely varying sulfur dioxide content have therefore already been undertaken in this laboratory.

The antiscorbutic vitamin content of the sulfured dried peaches used in this study is as high as that of any other foods yet reported, 1 gm. daily being sufficient to protect standard guinea pigs from scurvy over a period of at least 90 days. For similar protection there are required 1.5 to 3 gm. of oranges or lemon juice, or fresh tomato, 3 times as much banana, 6 times as much raw apple or pear or cooked potato, and nearly twice as much pineapple.

SUMMARY.

1. Peaches of known origin, namely, fresh, sun-dried, and dehydrated, both sulfured and unsulfured, were tested for vitamin C content.

2. The sulfured peach products retained the full antiscorbutic vitamin content of the fresh fruit, but the unsulfured sun-dried and dehydrated peaches retained no detectable amount of this property. The suggested possible relation of vitamin C protection to a minimum sulfur dioxide content or acidity is now under investigation.

3. The sulfured dried peach preparations were found to rank with orange juice, raw tomatoes, and other highly potent antiscorbutic foods.

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THE REACTION BETWEEN NITROUS ACID AND CERTAIN AMINO ACIDS AND RELATED COMPOUNDS AT 45°.

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In his original communication dealing with the reaction between amino acids and nitrous acid, Van Slyke (1) showed that while the majority of the naturally occurring amino acids when treated with nitrous acid yield their nitrogen quantitatively at room temperatures, glycocoll and cystine yield more than the theoretical amount of nitrogen. Later, Levene and Van Slyke (2) demonstrated that the amount of gas given off by glycocoll increased slowly with time, so that at the end of about half an hour the estimated amount of nitrogen was found to be 112 per cent. On the other hand, Van Slyke (3) found that the amount of gas given off by leucine in 10 minutes did not differ materially from the amount which was evolved in 4 minutes. Van Slyke (1) also showed that as much as 135 per cent of nitrogen was obtained when glycyl-glycine was treated with nitrous acid for 1 hour. Leucyl-glycine and leucyl-leucine did not react abnormally. No nitrogen was evolved when glycolic acid was treated with nitrous acid. With the exception of guanosine, the purine and pyrimidine derivatives were found to react normally.

Wilson (4) studied more extensively the reaction between purines and pyrimidines and certain related compounds and nitrous acid, and found that certain of them are abnormal with respect to their expected behavior towards nitrous acid. Skraup and Hoernes (5), Lewis and Updegraff (6), and others (7) believe that under certain conditions the reaction between certain amino acids and nitrous acid may proceed further than the stage of deaminization.

In attempting to explain the abnormal behavior of glycocoll

and glycyl-glycine, Van Slyke (1) after ruling out the possibilities that the extra gas may be due to the action of nitrous acid on glycolic acid, the expected end-product when nitrous acid acts upon glycocoll, and from the peptide nitrogen of glycyl-glycine by hydrolysis, concludes that the diazo compounds first formed do not decompose entirely in the normal way to yield glycolic acid or glycolyl-glycine but a portion of the reaction proceeds in such a manner as to lead to a disintegration of the molecule. As a result of the decomposition, some of the peptide nitrogen of glycyl-glycine is either set free or exposed so that it may be acted on by nitrous acid and eventually contribute to the gas which is measured. Van Slyke offers no explanation regarding the abnormal behavior of cystine. He states that by treating the gas with cuprous chloride to absorb a trace of carbon monoxide, he was unable to reduce the gas volume to the theoretical.

It is evident that since the total nitrogen of glycocoll and of cystine is in the form of free amino groups, any extra nitrogen which may be given off when they are treated with nitrous acid must come from the decomposition of the latter substance. The fact that carbon dioxide is given off in the decomposition of glycocoll and glycyl-glycine suggests that a part of the action of nitrous acid may be one of oxidation. Van Slyke (1) found that somewhat more gas is given off when cystine is treated with nitrous acid than when glycocoll is similarly treated.

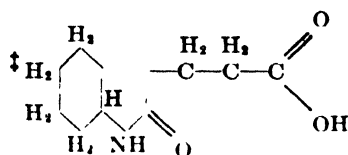
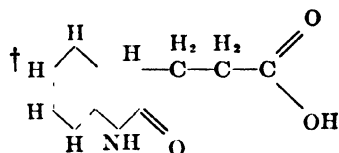
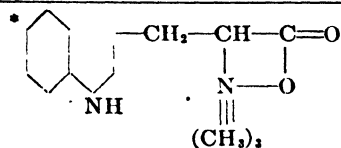
It interested us to study the reaction between certain amino acids and related compounds further in order to obtain more data relative to the compounds which were studied by Van Slyke. It also interested us to determine the effects of a higher temperature on the reaction between nitrous acid and certain amino acids. The experiments were carried out at 45°. This temperature was chosen in order to accelerate if possible any abnormal reaction which may take place. Lewis and Updegraff (6) showed that at this temperature practically all of the tyrosine content of casein is destroyed in an hour. In their experiments the Millon reaction was one of the tests used to follow the destruction of tyrosine. The disappearance of the Millon test may, however, indicate merely the introduction of nitroso groups into the phenyl ring rather than a complete destruction of the tyrosine molecule. Thus, it has been shown by Wheeler and Mendel (8) that diiodotyrosine

TABLE I.

Time Effect on the Reaction between Nitrous Acid and Certain Amino Acids and Related Compounds.

Temperature, 45°.

Substance.	Per cent of nitrogen after:			
	4 min.	15 min.	30 min.	60 min.
Amino acids.				
Alanine.....	98		100	99
β -Alanine.....	99	99	99	99
Arginine.....	108	118	132	150
Cysteine.....	113	122	122	128
Cystine.....	111	124	127	131
Glutamic acid.....	100	99	101	101
Glycocoll.....	108	113	115	117
Histidine.....	102		106	109
Norleucine.....	100		100	100
Ornithine.....	98	101	101	101
Oxyproline.....	0		0	0
Phenylalanine.....	100	100	99	100
Serine.....	105		108	105
Tryptophane.....	118	158	168	194
Tyrosine.....	99	103	106	113
Peptides.				
Glycyl-glycine hydrochloride....	127	130	130	
Leucyl-glycine.....	104	103	105	110
Related substances.				
α -Dihydroxy- β -dithiodipropionic acid	3.	8	11	13
Betaine of tryptophane.*.....	0.9	1.6	2.2	2.6
Formic acid.....	0		0	0
Glucose.....	0		0	0
Glycolic acid.....	0		0	0
Oxyindole Sample 1.†.....	49	87	100	99
“ “ 2.‡.....	0	0	0	0
<i>p</i> -Hydroxyphenylacetic acid.....	0		0	0
Sodium oxalate.....	0		0	0



does not give the Millon reaction. All apparatus and reagents were kept in an air bath which was under thermostatic control. The permanganate solution was changed frequently to insure complete absorption of the gases. The reacting solutions were shaken continuously for the time indicated in Table I. The nitrogen conversion factors were calculated with the aid of the equation given by Sharp (9). All amino acids used were recrystallized products and whenever analyses were not available, estimations of amino nitrogen were carried out at room temperatures. All solutions were made equivalent to a glyocoll solution containing 400 mg. per 100 cc. On account of the manipulations, it was found impossible to keep the temperature as constant as desired. The analytical errors may be as great as 2 per cent.

The results are presented in tabular form in Table I. The data confirm the observations of Van Slyke that glyocoll, cystine, and glycyl-glycine yield an abnormal amount of gas when treated with nitrous acid. At the temperature employed, tryptophane yields more nitrogen than can be accounted for on the basis of the amino group. In fact, the data indicate that in an hour's time, tryptophane yields almost its total nitrogen content. About 18 per cent of the second nitrogen group is set free in 4 minutes. At 22° only the expected amount of nitrogen was given off in 4 minutes, while in 30 minutes the yield was 110 per cent and in 60 minutes, 116 per cent. The unsaturated oxyindole compound yields its nitrogen completely within a period of 30 minutes, while the saturated compound does not react. The betaine of tryptophane reacts only slightly. It is not surprising to find that the indole nitrogen of tryptophane and of the unsaturated oxyindole compound is set free. Kendall and Osterberg (10) have shown that both indole and isatin yield 7 to 12 per cent of their nitrogen in about 4 minutes. Arginine yields more nitrogen than can be accounted for on the basis of the amino group. The amount increases with time. This indicates that the guanidine group is slowly attacked by nitrous acid. This was also noted by Sekine (11). Serine, and particularly tyrosine and histidine, yield slightly more than the expected amount of nitrogen whereas alanine, glutamic acid, norleucine, ornithine, and phenylalanine react normally. No nitrogen is given off by oxyproline. Glycyl-glycine, as noted by Van Slyke, yields more than the expected amount of nitrogen.

Leucyl-glycine yields slightly more than the theoretical amount of nitrogen; the value is much less than that of glycyl-glycine. It does not seem probable that the extra gas obtained from leucyl-glycine comes from the glycyl radical. Since the amount increases slowly with time, it is possible that a slight amount of hydrolysis takes place. Fischer and Koelker (12) obtained more than the calculated amount of gas on treating leucyl-serine with nitrous acid at 60°. Under the same conditions of experimentation, glycyl-leucine yielded considerably more gas than the amount obtained from leucyl-serine. Formic acid, glucose, sodium oxalate, and glycolic acid yielded, within the limits of error, no gas.

It appears that the abnormal behavior of cystine and cysteine can be connected with their sulfur radicals. When cystine is shaken for several minutes either at room temperature or at 45° with nitrous acid, several drops of barium chloride solution being added to the mixture, a precipitate of barium sulfate forms which increases in amount as the shaking is continued. 40 mg. of cystine were treated with nitrous acid in the presence of barium chloride and the mixture allowed to stand at room temperature for about 20 hours. 56 per cent of the cystine sulfur was recovered as barium sulfate. The reagents were free from sulfate ions. In Table I data are given which show that gas is evolved when α -dihydroxy- β -dithiodipropionic acid is shaken with nitrous acid, the amount increasing with time during the period of 1 hour. This substance was prepared from cystine according to the directions given by Westerman and Rose (13). The product is not entirely pure. It is difficult to rid it of a small amount of yellow color. The color is indicative of the presence of a nitrogenous group, possibly the nitroso. Dunn and Lewis (14) believe that the light yellow color of deaminized casein is due to the presence of nitroso groups.

In attempting to account for the abnormal behavior of glycocoll, a number of hypotheses might be advanced. It is evident that the extra gas cannot, as Van Slyke has shown, be due to the reaction of nitrous acid on glycolic acid, the expected end-product of the reaction. Neither is it due to the action of nitrous acid on formic acid or oxalic acid, substances which might conceivably be formed from glycocoll by oxidation. Practically the same amount of gas was obtained from solutions containing 2 equivalents of glycolic acid in addition to the glycocoll as was found for glycocoll

alone. It is not fully established that the gas which is measured is wholly nitrogen. It is conceivable that in a reaction involving decomposition of nitrous acid, nitrous oxide may be given off. Such reactions are known (15). However, it does not appear probable that the extra gas measured is nitrous oxide since this substance is quite soluble in water. We found practically no difference in our glycoll estimations when fresh permanganate solution and fresh water in the burette and leveling bulb were employed. It is evident that when glycoll is treated with nitrous acid at least two reactions, one fast and one somewhat slower, take place. The first reaction probably involves the formation of the diazo compound and its subsequent decomposition, yielding glycolic acid and nitrogen. A not considerable portion of the reaction does not proceed altogether this way. It would be possible to postulate a number of intermediate compounds but in the absence of positive identification, this had better not be done. The decomposition of this intermediary compound which, for the sake of illustration only, may be compared to nitrosyl-sulfuric acid, results not only in the breakdown of the glycoll molecule with the formation of carbon dioxide but also in the setting free of unabsorbed gas which probably is nitrogen. The reaction is one of oxidation involving the formation and subsequent decomposition of an intermediary compound. This is conditioned upon the presence of an amino group in the molecule which is acted on. Probably a similar type of reaction takes place when cystine sulfur is oxidized by nitrous acid. When the amino group of glycoll is protected, as in leucyl-glycine, this intermediary compound probably does not form.

In Fig. 1 the results which were obtained by Van Slyke and by ourselves with a number of substances have been plotted. A number of points have been included in certain of the curves, the data for which are not given in Table I. It is evident that the amount of gas which is given off by glycyl-glycine at any time after the first 2 minutes is considerably greater than that yielded by glycoll in the same time. This is probably due, as Van Slyke has pointed out, to the decomposition of some of the glycolyl radical, thus setting free a certain amount of nitrogen from the peptide linkage. The evolution of gas from the cystine and cysteine reactions proceeds much more slowly than in the case of glycoll and glycyl-glycine.

The contrast in the behavior of the two oxyindole compounds is noteworthy. The saturated compound is resistant to the action of nitrous acid. If the breakdown of the unsaturated compound and the liberation of the indole nitrogen from tryptophane are due to oxidation by nitrous acid it does not, however, result in the

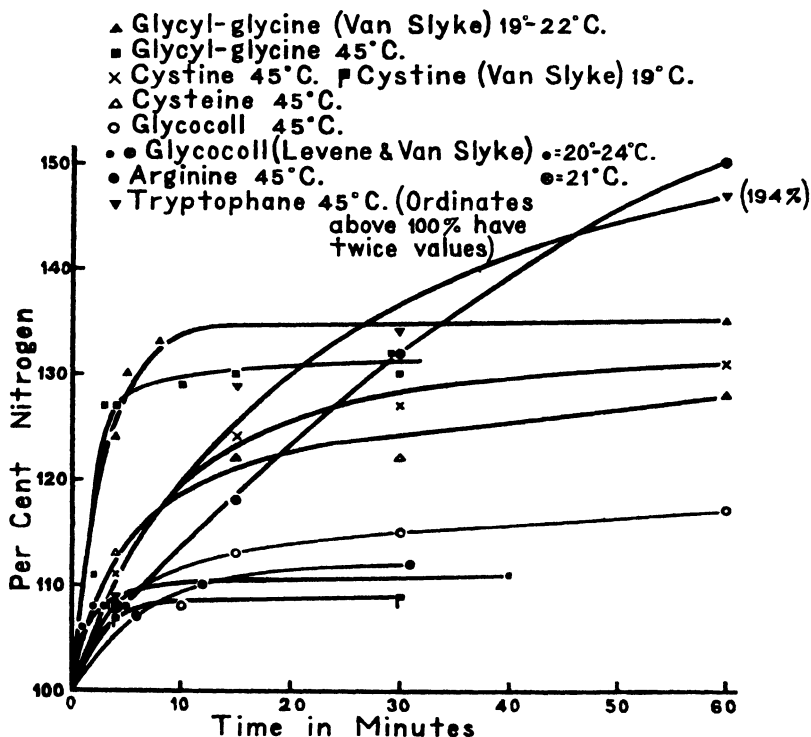


FIG. 1.

setting free of any extra nitrogen as, for instance, in the decomposition of cystine. It is also possible that the reaction is one of hydrolysis. Since the betaine derivative of tryptophane is very much more resistant to decomposition by nitrous acid, it appears that deaminization is an essential prerequisite to further extensive decomposition of the molecule.

The oxyindole compounds were kindly supplied to us by Dr. E. C. Kendall of the Mayo Foundation, and the betaine derivative of tryptophane by Mr. R. W. Jackson of Yale University.

SUMMARY.

1. The reaction between nitrous acid and certain amino acids and related compounds at 45° has been studied.

2. It is found that glycocoll, glycyl-glycine, cystine, cysteine, tryptophane, arginine, and an unsaturated oxyindole derivative yield more nitrogen than can be accounted for on the basis of the free amino groups. Other substances tested were found to react normally.

3. Explanations as to the mechanisms involved in the reactions which yielded abnormal amounts of gas are given.

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THE STRUCTURE AND COMPOSITION OF HEMOSIDERIN.

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INTRODUCTION.

In the spleen, liver, and kidneys of most mammals may be found varying quantities of yellow-brown granules and to these granules has been given the general name hemosiderin. Since the formation and location of hemosiderin coincides in time and place with the destruction of blood, the idea has become firmly established that hemosiderin represents some step in the disintegration of hemoglobin and the formation of bile pigment. The question has come under consideration in many connections throughout the fields of physiology and clinical medicine. The great range of literature in which there is mention of hemosiderin may be seen in the reviews of Whipple (1), Rous (2), and Rich (3). These papers summarize and discuss the present status of the problems concerning the normal and abnormal destruction of red blood corpuscles and formation of bile pigment. It is clearly brought out that hemosiderin is in some way connected with these processes but the extensive although scattered evidence does not conclusively indicate the exact nature of the reactions involved. Nor is much known of the constitution of the substance itself save that it contains iron in some form. The fact that hemosiderin has a high iron content has led many investigators to conclude that in the breakdown of hemoglobin the iron is split off from the hematin as hemosiderin and the iron-free remainder of the molecule goes to make up the bile pigment (*cf.* Rich (3)). This view has found its way into the text-books (such as Wells, "Chemical Pathology"). It has seemed desirable to omit entirely all con-

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sideration of the source or function of hemosiderin and to confine this investigation solely to the question of its actual composition.

The attention of investigators has been centered principally on the fact that hemosiderin contains a large amount of iron. That this is true has been often demonstrated by various means, both on the granules *in situ* and on various tissue preparations (cf. Abderhalden (4), Oppenheimer (5), Wells (6), Muir and Dunn (7), McMaster, Rous, and Larimore (8), Muir and McNee (9), Boycott and Douglas (10), Brown (11), Oberzimmer and Wacker (12), etc.). If it is assumed, therefore, that iron is present in the hemosiderin granules, the most important question chemically is this: in what form does the iron exist? Several opinions have been expressed, of which the more representative are these:

1. The iron is combined with an organic molecule. Brown (11, 13) thinks that hemosiderin is an iron-protein compound.

2. The iron is inorganic. The brown color of the granules and the ease with which the color is extracted with acid lend support to the idea that the granules are masses of ferric (or possibly also ferrous) oxide or hydroxide. One of the chief exponents of this view is Fischer (14). He says, "He [referring to Hueck¹] emphasizes that if one treats sections containing hemosiderin with acid it is possible to demonstrate the presence of iron, and with sufficiently large quantities to precipitate with soda hydrated iron oxide, 'a body which resembles hemosiderin in nearly all respects.'" Fischer says later, "regarding hemosiderin, since it cannot be extracted by water, it is most probable that it is nothing other than finely divided elementary iron, overcast with a layer of oxide which gives the pigment its color."²

Aside from the fact that no evidence of any metallic iron can be discovered, the experiments reported here tend to confirm the view that in hemosiderin the iron is inorganically bound.

EXPERIMENTAL.

The material used was horse spleen, fresh from the slaughterhouse, which was selected because of its high hemosiderin content. Preliminary analyses showed that the hemosiderin and iron

¹ Hueck, *Pigmentstudien*, Habilitationsschrift, Munich, 28-29 (1912).

² Translated by the author.

content run parallel. The cells are usually packed full of granules and in such cases the iron may be as much as 4 per cent of the fresh weight of the spleen. For work on the granules in the tissue under the microscope, sections were made with the microtome from frozen blocks of the fresh organ. They were then washed several times in distilled water to remove what blood might remain. This procedure obviates any disturbance due to fixation or preservatives. The larger granules correspond roughly in size to red blood corpuscles; the smaller are barely visible under the ordinary high power. The method used for obtaining them free from the tissue is described below.

Chemical Examination of Granules.

1. *Properties of Acid Extract of Granules.*—0.5 gm. of tissue was cut into thin sections and treated with 60 cc. of 30 per cent HCl. It was centrifuged and the clear liquid used for the following tests: (a) NaOH gives a brown precipitate of $\text{Fe}(\text{OH})_3$. (b) $\text{K}_4\text{Fe}(\text{CN})_6$ gives a heavy precipitate of ferric ferrocyanide. (c) KCNS gives a characteristic deep red color. (d) $(\text{NH}_4)_2\text{S}$ gives a heavy black precipitate. These four qualitative tests all show the typical reactions of ferric iron. The extract with strong HCl, therefore, contains the iron in simple ionic form.

2. *Reactions of Granules in Tissue.*—Thin sections of fresh tissue were treated with the same reagents with the following results: (a) NaOH has no apparent effect whatever on the granules. (b) $\text{K}_4\text{Fe}(\text{CN})_6$ + dilute HCl causes the granules to turn a blue-green which becomes true Prussian blue on warming (cf. Oberzimmer and Wacker (12)). This reaction is not typical. Free ferric iron should turn deep blue immediately. (c) KCNS + HCl causes a general, diffuse, faint coloration of the entire tissue after about 30 minutes, but there is no localization of color in the granules. The general colorization may come from free iron present either as an impurity (e. g. from the section knife) or from the slow action of the dilute HCl on the granules. The striking point in this experiment is that there is no characteristic coloration due to the iron in the granules themselves. (d) $(\text{NH}_4)_2\text{S}$ causes intense blackening of the granules. The black may be removed by dilute acid, with the evolution of H_2S gas, indicating that the black substance is FeS which is formed from ferric salts by reduction. (e) $\text{K}_3\text{Fe}(\text{CN})_6$

has no effect whatever. The total absence of Turnbull's blue suggests that there is no ferrous iron present, although the anomalous behavior of the ferric reactions makes it impossible to insist on the point.

Of the first four reagents only one, ammonium sulfide, reacted in a typical manner. The most interesting deviation from what might be expected is the failure to get a good color with thiocyanate, since this is one of the most delicate known tests for ferric iron. The presumption therefore is that the iron in the granules is not in the ionic form. But free ferric iron is obtained in the liquid when the granules are extracted with strong acid. The effect of the latter must be to remove the iron from an organic compound if there is one, or to alter some molecular to the ionic state.

3. Reactions of Granules after Extraction with Acid.—10 per cent hydrochloric or sulfuric acid causes the hemosiderin to bleach and become colorless in a few minutes. With weak acid a longer treatment in the cold, or a brief boiling, brings about the same bleaching of the tissue. After extraction the customary colors are not obtained with ferrocyanide or ammonium sulfide; in other words there is no reaction for iron at all. The latter is evidently quantitatively removed from the granules by the acid. The characteristic color of the granules is destroyed in the process.

In order to confirm the above results it seemed advisable to try to separate the granules from the mass of tissue in which they are embedded. For it is sometimes difficult to distinguish the granules from the cells around them, especially after bleaching, and furthermore no quantitative work can be done in the presence of a great quantity of organic debris. Several methods were tried, of which the following gave the best results. From a fresh spleen the pulp was scraped out and digested in the cold for several days with 3 per cent sodium or potassium hydroxide. (Previous examination had shown that digestion with alkali did not alter the characteristic reactions of the granules.) After the proteins had been hydrolyzed the somewhat syrupy fluid was centrifuged and the sediment washed (by centrifuging) several times with 3 per cent soda or potash and then with distilled water. The product was a suspension of hemosiderin almost entirely free from organic matter. The effect of digestion with concentrated alkali will be discussed in another connection.

The experiments reported above were now repeated and in every instance the results were the same; there was a blue-green color with ferrocyanide and no color with acid thiocyanate. This demonstrated that the hemosiderin had not been essentially altered during the digestion and washing.

As it was now possible to follow the behavior of the granules, unobscured by any extraneous material, the following observations were made relative to their structure.

1. It has been stated above that strong HCl completely removes the iron and decolorizes the granules. This decolorization was now carefully followed under the microscope. In the acid the granule slowly loses its brown color, becomes yellow, and ultimately entirely colorless. In the final stage it has the appearance of a ghost in that it retains its original size and shape. Although hyaline and almost transparent it remains the definite body it was in the first place.

2. At various stages of the bleaching the granules were treated with ammonium sulfide, which reacts with any iron present to give a black or gray color. In the early stages the black is merely less intense, but as the general fading continues one gets a less and less intense darkening with sulfide until finally this substance has no effect whatever. In many cases, however, the black appears as localized regions *within* the granule. There may be one or several of these regions in the interior, the periphery being colorless. The larger granules tend to exhibit this phenomenon more than the smaller.

From these data the conclusion may be drawn that hemosiderin granules have a definite physical structure, and are not irregular particles of a homogeneous chemical compound, such as ferric hydroxide. For were they the latter it is to be expected that removal of the iron by HCl would break up or alter the structure of the molecules and completely disintegrate the particle. At least there would be some change in the size or shape of the particle. It is more reasonable to suppose that the granule consists of a mass of inert (organic) material in some way heavily impregnated with an iron compound, and that the acid chemically or physically leaches out the iron, as it were, starting with the periphery and eventually penetrating to the deeper parts. The unevenness which characterizes the removal of iron (as indicated by ammo-

nium sulfide) may be due to variation in the physical dimensions, or the density, of the granule or possibly may be due to differences in the concentration of the iron within.

Hydrochloric acid in all concentrations removes the iron, as ferric chloride, from hemosiderin but some other acids behave very differently, especially nitric and acetic acids, as the following series of experiments shows. In all the analyses for iron the substance was evaporated to dryness, ignited, and taken up with H_2SO_4 . The iron was reduced by zinc metal and titrated against standard permanganate.

TABLE I.
Effect on Hemosiderin of Various Acids.

Sample A.	Sample B.	Sample C
Placed in 10 per cent HCl and allowed to stand 20 hrs.; centrifuged and fluid decanted.	10 per cent HNO_3 . Same as for Sample A.	10 per cent CH_3COOH . Same as for Sample A.
Fluid. Clear, yellow; 3.35 mg. Fe.	Clear, colorless; 2.5 mg. Fe.	Slightly opalescent semicolloidal deep red-brown; 4.35 mg. Fe.
Residue. Treated with 5 cc. distilled water and allowed to stand 1 day; centrifuged; fluid colorless and clear; 0.82 mg. Fe.	Treatment same. Fluid yellow-brown opalescent; 1.87 mg. Fe.	Treatment same Fluid clear and colorless; 0.45 mg. Fe.

Three lots of hemosiderin, each containing 5 mg. of iron, were placed in three test-tubes and the procedure adopted as shown in Table I. In each case the difference between 5 mg. of iron and the total quantity shown represents that contained in the second residue which was not extracted by acid.

The HCl removes the iron in ionic form and apparently the HNO_3 at this concentration also does so. But the fact that when the residue from HNO_3 is placed in distilled water a brownish solution appears, suggests the possibility that (1) the 10 per cent nitric acid did not remove all the iron (also indicated by the iron analyses)

or that (2) more dilute nitric acid might take out the iron in a different form. The acetic acid removed almost all the iron from the granules but not in the same manner as the other two acids. Rather the extract from 10 per cent acetic acid resembled the distilled water extract from the original 10 per cent nitric acid residue. Therefore it was necessary to investigate more closely the concentration relations of the two acids.

(a) *Nitric Acid*.—Suspensions of hemosiderin were made up with different concentrations of HNO_3 and the color of the extract observed. The results, which are given in Table II, show that nitric acid in great dilution has no effect on hemosiderin, in medium concentration (0.05 to 1 per cent) it extracts a red-brown pigment,

TABLE II.
Effect on Hemosiderin of Different Concentrations of Nitric Acid.

Concentration of HNO_3 per cent	Extract.
10	Colorless, good test for ferric iron.
4	Same.
2.5	Slightly yellow.
2.0	Quite yellow.
1.5	Orange.
1.0	Red-brown, slightly opalescent.
0.4	Same.
0.05	"
0.005	Colorless, very faint test for ferric iron.

and in higher concentration it behaves like hydrochloric acid and removes ferric iron.

(b) *Acetic Acid*.—A suspension of hemosiderin which by analysis was found to contain 10 mg. of iron per cc. was divided into four lots of 1 cc. each. Each sample was then made up to a definite concentration of acetic acid. From the results in the tabulation

	Sample A.	Sample B.	Sample C.	Sample D.
Acid, per cent.....	2	0.5	0.1	0.01
Color of extract.....	Red-brown.	Light brown.	Pale yellow-brown.	Colorless.
Fe in extract, mg.....	7.6	6.5	2.8	Trace.

it may be concluded that acetic acid extracts the pigment and also the iron in proportion to the concentration of the acid but that high concentrations do not break up the pigment into ferric iron.

The question next arises whether the iron found by analysis of the extracts is contained in the pigment or is free in solution. In other words can the iron and the pigment be separated and if so to what extent?

Use was made of the fact that the pigment which is extracted by 1 per cent HNO_3 is precipitated by 20 per cent HNO_3 . This precipitate may be centrifuged off and dissolved in distilled water. If the iron is not in the pigment, it will be removed by this process and we shall eventually arrive at a point where the pigment will be free from iron. If it is in the pigment, then as long as we have the latter we shall be able to find iron on analysis.

A suspension of hemosiderin was made up in 1 per cent HNO_3 such that each 10 cc. of the suspension contained 10 mg. of iron. The acid extracted the pigment, and the following procedure was adopted. (a) A solution of 100 cc. of pigment was centrifuged. 10 cc. were taken for analysis and found to contain 7.7 mg. of Fe. The remaining 2.3 mg. of Fe were left in the granules, the extraction being incomplete. (b) 20 cc. of 20 per cent HNO_3 were added and the precipitate centrifuged. An analysis of 12.2 cc. of the fluid gave 1.5 mg. of Fe. Either it had originally existed in the ionic state in the granules, or it represented decomposed pigment. In either case there should remain 10—3.8 or 6.2 mg. of Fe in each 10 cc. of pigment. The precipitate was dissolved in 90 cc. of water, and 10 cc. of the solution contained 6.3 mg. of Fe. (c) 20 cc. of 20 per cent HNO_3 were added and the precipitate centrifuged. This time analysis of the fluid gave only a trace of iron. The precipitate was dissolved in 80 cc. of water and 10 cc. of this contained 5.7 mg. of Fe. (d) The process was repeated. The fluid again contained only a trace of iron, whereas the pigment yielded 6.3 mg.

After the first precipitation, therefore, all the iron remains with the pigment. If the latter were broken up by the acid, it would gradually lose its iron on repeated precipitation, but such is not the case. Or if it contained no iron at all, none of that element would be found after two or three analyses. Furthermore with hydrochloric acid all the iron appears as ferric chloride and there is

no sign of the pigment. The hemosiderin granules thus apparently contain an iron pigment *along with* a small amount of free ferric iron.

The next step was to investigate the chemical nature of the red-brown pigment. This was done in four ways.

1. *Qualitative Reactions*.—A solution of the pigment, extracted with nitric acid and freed from ionic iron by precipitation, was treated in the test-tube with the same reagents which had previously been used on the granules. (a) NaOH has no effect; *i.e.*, it forms no precipitate of $\text{Fe}(\text{OH})_3$. (b) $\text{K}_4\text{Fe}(\text{CN})_6$ gives a blue-green coloration, turning to Prussian blue on warming. Ferric ferrocyanide is then precipitated. (c) KCNS gives a faint, barely perceptible, coloration, such as might be caused by the traces of free iron present. An equivalent amount of iron in the form of FeCl_3 gave an intense red color. (d) $(\text{NH}_4)_2\text{S}$ gives a heavy black precipitate of ferric sulfide.

These reactions correspond precisely to those observed in the granules and furnish good evidence that we are dealing with one and the same substance, both in the extract and in the granules themselves. Other tests for iron were applied such as those with tannin, phosphate, pyrogallol, and H_2O_2 , etc., and were checked with standard ferric salts. In every case the pigment reacted not at all, or in an abnormal manner. Tests for ferrous iron were entirely negative.

2. *Quantitative Analysis*.—The iron content of the pigment (and granules) is very high. The following analyses give an idea of its order of magnitude. The material was evaporated to dryness at 100° and ignited. The iron was determined by the permanganate method.

Granules.

Sample No.

1	Dry weight = 31	mg.	Iron = 4.7 mg. or 15.1 per cent.
2	" " = 24	"	" = 4.3 " " 18 " "
			Average = 16.5 per cent iron.

Pigment.

Sample No.

1	Dry weight = 15.5 mg.	Iron = 7.7 mg. or 49.5 per cent.
2	" " = 9 "	" = 4.1 " " 45.5 " "
3	" " = 20.4 "	" = 7.8 " " 38 " "
7	" " = 11.1 "	" = 4.7 " " 42.5 " "
		Average = 44 per cent iron.

In view of the peculiar reactions of this substance, it was important to know whether the iron was organically or inorganically combined. Now if a definite organic compound of iron were present, it would necessarily be of low molecular weight. For if 1 atom of iron per molecule is assumed, and the proportion of iron 45 per cent, the molecular weight would be about 125. If there were just 1 atom of carbon per molecule then the substance would have to contain at least 10 per cent carbon. Similarly there would have to be 11 to 12 per cent nitrogen, were there any at all. Accordingly a complete organic analysis was made on samples of the pigment as extracted by both nitric and acetic acids.³ The nitric acid extract was found to contain: Fe 55 per cent, C 1 per cent, H 12.5 per cent, O 27 per cent, and N less than 0.1 per cent. Analysis of the acetic acid extract gave approximately the same result save only that the carbon content was slightly higher due to traces of acetic acid remaining in the solution.

These analyses were twice checked with respect to nitrogen. Two analyses were made by the writer in the Cambridge laboratory, using a micro-Kjeldahl method, and only slight traces of nitrogen found. Two more were made by Mr. George Giragosintz of the Department of Biochemistry, University of California. Here different material was used but no significant amount of nitrogen could be detected.

The conclusion may reasonably be drawn from these analyses that the pigment contains neither carbon nor nitrogen. Likewise it is exceedingly difficult to assume that the iron in hemosiderin itself is organically combined. For the iron of hemosiderin has been shown to be nearly all in the pigment and the pigment does not contain an appreciable amount of carbon or nitrogen.

3. *Precipitation Reactions.*—The pigment when dissolved in distilled water can be precipitated by nitric acid in moderately high concentration, as has been described. This precipitate can be redissolved in water. There are also other methods of precipitation which indicate that the material is in a state of physical instability. (a) When an aqueous solution is evaporated the residue is an amorphous brown mass which is insoluble in water, acid, alkali, ether, chloroform, and all other substances tried. It is

³ Analysis made by Dr. A. Schoeller, Feinchemie, Berlin-Schmargendorf.

soluble in hot dilute, or cold concentrated HCl but is thereby converted into the ferric salt of the acid. It thus behaves under these conditions as a hydrophobe colloid. (b) The pigment may be precipitated with formic acid (20 per cent). This precipitate is soluble in water. (c) It may be brought down with a mixture of alcohol and ether. This precipitate is soluble in water only with difficulty. (d) It may be brought down by a neutral solution of sodium acetate, sodium chloride, and other salts. In these cases it may or may not be redissolved by water. (e) If a precipitating reagent (*e. g.* formic acid, sodium acetate) be added very carefully to a solution of the pigment, the very faint opalescence of the solution becomes more marked. A definite Tyndall effect may also be observed. If it be then centrifuged 5 or 10 minutes at high speed (9000 R. P. M.), there will be a difference between the upper and lower portions of the tube. The bottom will be relatively cloudy and deeper colored, whereas the top will be clearer and lighter colored. (f) Mention should be made here of the behavior of the granules when they are separated from the tissue by concentrated alkali. The experiments hitherto reported were all made on hemosiderin which had been digested in 3 per cent alkali. The product of digestion in stronger alkali is the same as that in the weaker because all the chemical reactions are the same (iron reactions, etc.), but the physical properties appear to differ.

In this instance the red-brown pigment is extracted not by dilute acid but by distilled water itself. If the sediment is washed clean of organic matter in an alkaline medium and is then put in distilled water, the pigment is removed. This extract has the appearance of the acetic acid extract previously discussed. The pigment is, however, precipitated by dilute acid, dilute alkali, or neutral salts, and in no case can it be redissolved in distilled water. The acid precipitate is not soluble in alkali, nor the alkali in dilute acid. It cannot be dissolved in anything short of strong HCl, which of course decomposes it. The difference in coagulability between these two extracts seems to be due to the difference in concentration of the alkali used in digestion.

These six sets of observations, although purely qualitative, make it probable that we are dealing with iron in a colloidal form. The appearance of the solution, the extreme ease of coagulation, the erratic behavior in the presence of inorganic ions, all point to an existence of a colloid.

4. *Some Reactions of Artificial Iron Salts.*—Since hemosiderin in the granule and as an extract reacts in a peculiar manner when treated with certain reagents and since there is evidence that hemosiderin may be of a colloidal nature, it seemed worth while to try the effect of these reagents on a known colloidal solution of ferric iron. The ferric oxide sols (*cf.* Weiser (15)) are very numerous and differ in their properties according to their mode of preparation. One of the best known and most easily prepared is that obtained by precipitating a dilute solution of ferric chloride with a base. This method was accordingly used, and a heavy precipitate of so called ferric hydroxide obtained. The experimental procedure from this point resembled that with hemosiderin, as the following examples indicate.

Experiment 1.—An iron solution was made by running 10 per cent NH_4OH into 5 per cent saturated FeCl_3 till very slightly alkaline. The precipitate was washed several times by decantation. Acetic acid was added to a concentration of 2 per cent. A red-brown solution was obtained in 6 hours. The solution was then centrifuged to remove undissolved precipitate. The fluid was clear brown. It gave a green color with potassium ferrocyanide and only slight traces of color with potassium thiocyanate. The residue, dissolved in water, gave an opalescent, brown solution, which became green with ferrocyanide, and showed no color with thiocyanate.

Experiment 2.—A precipitate of ferric hydroxide was treated with 2 per cent lactic acid. A brown solution was obtained in 3 hours and was precipitated with 20 per cent nitric acid. The precipitate was centrifuged. The fluid became a deep red color with thiocyanate. The residue, dissolved in water, gave a light brown solution but only a very slight color with thiocyanate.

Experiment 3.—A precipitate of ferric hydroxide was treated for 15 hours with 2 per cent acetic acid. A red-brown solution was obtained, which was centrifuged to remove undissolved precipitate. (a) The fluid gave a green color with ferrocyanide, and showed slight traces of color with thiocyanate. (b) When precipitated with 20 per cent nitric acid and centrifuged, the fluid was clear and colorless. It gave some color with thiocyanate, representing iron which had been ionized by the acid. The residue from (b) dissolved in water as a clear, brown solution.

This turned green with ferrocyanide, and gave *no* color whatever with thiocyanate. The residue from (a) was soluble in water. It became green with ferrocyanide and gave *no* color with thiocyanate.

Although these experiments differ somewhat in detail from the previous ones with hemosiderin, the same general procedure was followed and the same general results were obtained. Particularly worthy of note are the facts that in both cases the original substance (hemosiderin or ferric hydroxide) may be got into a brown solution by means of dilute acids (*e. g.* acetic), may be precipitated therefrom by means of stronger acids (*e. g.* nitric), and in the sol state may both give a green color with ferrocyanide, and no color or only traces of color with thiocyanate. The analogy between the reactions of hemosiderin and those of a ferric oxide solution is thus very striking and taken together with the preceding evidence makes it very probable that hemosiderin is itself simply ferric oxide.

DISCUSSION.

It is clear from the foregoing that hemosiderin is not a definite chemical entity, such as hemoglobin. The granules are of no particular shape or size but are analogous to red blood corpuscles in that they form a substrate on which a chemical compound is deposited. Possibly they may carry the red-brown, iron-containing pigment in some such fashion as the corpuscles carry hemoglobin. At any rate the pigment may be removed, leaving the substrate, or stroma, intact.

This pigment is of peculiar nature. It contains no carbon or nitrogen and therefore cannot be an organic compound. Its iron content is very high, and aside from iron consists only of hydrogen and oxygen (or water). It must be an inorganic compound of iron, yet its reactions are not those characteristic of ferric iron. It gives none of the reactions of ferrous iron whatever. Hence the iron must be in the form of an oxide, or hydroxide. The behavior of the material in solution suggests that it is in the colloidal state. Furthermore the behavior of a pure ferric oxide sol toward acids, and toward ferrocyanide and thiosulfate, has been found to coincide to a reasonable degree with that of the pigment. If this is so, then we may tentatively define hemosiderin as some form of colloidal ferric oxide, physically combined with an organic sub-

strate, the stroma of the granule. It may be adsorbed on the surface of the stroma but it is more likely that the latter is permeated by the iron compound, the molecules being held in place throughout the substance of the granule by physical forces. Were the iron held only on the surface it would be quickly removed by strong acid, but observation has shown that some of it persists in the interior and can there become blackened by sulfide after the surface is no longer affected by that substance. Another possibility is that the iron in the interior of the granule is in the form exhibited in the pigment but that the surface is covered with a thin layer of free ferric iron. This conception would coincide to a certain extent with that of Fischer, mentioned in the introduction. But there seems to be no metallic iron present. The problem of the exact method whereby the iron compound is held in the granule awaits further investigation.

In regard to the broader aspect of the question, the rôle of hemosiderin in the economy of the organism, it must be said that these experiments tend to substantiate the view that the iron of hemoglobin is split off from the organic part of the molecule, and is deposited, in an inorganic form, as hemosiderin.

SUMMARY.

1. Tests under the microscope demonstrate that hemosiderin consists of an iron compound which can be removed from the granules by treatment with acid, leaving the substrate practically intact.
2. The granules can be obtained free from tissue by a process involving digestion with alkali.
3. The iron-containing substance can be extracted and observed *in vitro*.
4. This material reacts with thiocyanate and other substances in a manner which is not characteristic of ionic iron.
5. This material has been found by analysis to contain only iron, hydrogen, and oxygen. It apparently exists as a colloid.
6. A pure ferric oxide solution has been found to react with thiocyanate and other substances in approximately the same manner as the extract of hemosiderin.
7. It is concluded that hemosiderin consists of organic granules impregnated with some form of ferric oxide.

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CALCIUM AND MAGNESIUM RELATIONS IN THE ANIMAL.*

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INTRODUCTION.

As a result of certain experiments concerned with the effect of the injection and ingestion of magnesium salts on calcium retention in animals, a prejudice against the therapeutic use of magnesium salts has arisen among certain medical authorities who correspondingly have advised against their use as antacids and laxatives. Likewise in livestock production the use of dolomitic limestone as a supplementary source of calcium for dairy cattle, swine, and chickens has been curtailed by the belief, often fostered by dealers in lime, that the magnesium of dolomite is detrimental. This prejudice is without adequate experimental support.

The idea of a physiological antagonism in nutrition between calcium and magnesium originated with Oscar Loew, who evolved this theory through experiments in plant nutrition (1) and later extended it to include also the nutrition of animals (2). The basis for his belief as applied to animal nutrition lay not in his own work but in the experiments of others, who had demonstrated a loss of calcium from the body on injection or ingestion of magnesium salts. Of the two methods, injection experiments have on the whole given the more positive evidence for the loss of calcium sometimes designated as the "washing out" effect of magnesium. This is to be expected since injection, especially intravenous and to a lesser extent subcutaneous injection insures the presence of the magnesium salts in the blood stream; whereas ingestion does not.

Mendel and Benedict (3), working with dogs, cats, and rabbits, noted an increased elimination of calcium in the urine when aqueous solutions of magnesium salts were injected subcutaneously, and in turn of magnesium

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when calcium salts were injected. Schiff (4) studied the action of subcutaneously injected MgSO_4 on the calcium and magnesium metabolism of the infant. He found a greatly increased calcium excretion in the urine and a small increase of calcium in the feces after the injection of 0.2 gm. of MgSO_4 per kilo of body weight. Later Schiff with his coworker Stransky (5) found this to be true not only for normal children, but also for children afflicted with "calciumuria" or abnormal excretion of calcium in the urine. Whelan (6) in 1925, injected solutions of the chlorides of calcium and magnesium into female dogs. An increase in urinary calcium followed the injection of MgCl_2 , but the effect of CaCl_2 on magnesium excretion was variable.

Stransky (7) studied the calcium and magnesium contents of the blood of rabbits into which MgSO_4 was injected subcutaneously in amounts sufficient to produce the narcosis first described by Meltzer and Auer (8). The result was a great increase in magnesium in the plasma and a definite lowering of the calcium. Stransky attributed the narcosis to the great lowering of the Ca:Mg ratio in the blood. Richter-Quittner (9) corroborated this work and made the interesting observation that, whereas normally only 50 to 60 per cent of the calcium salts in the blood are ultrafiltrable, after injection of magnesium salts 89 per cent were ultrafiltrable. Thus a change in the chemical combination of calcium in the blood seemed to be effected by the magnesium salts, which in this way may produce the "washing out" effect. The accumulated evidence leaves little doubt that the injection of magnesium salts results in a definite loss of calcium from the body.

Studies of the effect of the ingestion of magnesium salts on calcium retention give more variable results than those dealing with injection. Malcolm (10), in 1905, produced a small increase in calcium excretion in dogs by the feeding of MgCl_2 . Hart and Steenbock (11) fed MgCl_2 and MgSO_4 to swine and demonstrated an increased elimination of calcium in the urine, but not in the feces. They also made the observation that the feeding of soluble phosphates with the magnesium salts appreciably decreased the loss of calcium, an effect not due to a change in the path of elimination of the calcium, as might be supposed at first glance, for the fecal calcium showed only a very slight increase during this period. The results of Hart and Steenbock with swine have been strikingly corroborated by the recent work of Palmer, Eckles, and Schutte (12) with cattle. The daily ingestion of 155 to 165 gm. of Epsom salts ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) markedly lowered the calcium balance of cattle on phosphorus-deficient rations. Additional phosphorus in the ration overcame the detrimental effect of the magnesium salts.

Of interest in this connection are the recent experiments of Shipley and Holt (13) and of Kramer, Shelling, and Orent (14, 15). These investigators, using Shipley's ingenious technique in studies on the calcification of rachitic cartilage *in vitro*, showed that magnesium salts had a specific inhibitory action upon the deposition of the bone-forming elements in the

rachitic metaphyses. They showed further that additions of phosphate could overcome the inhibitory action of magnesium

Studies on humans have shown only slight and variable effects of magnesium ingestion. Givens (16) reports no effect on urinary calcium when foods high in magnesium or small amounts of magnesium citrate were ingested. Underhill, Honeij, and Bogert (17) found no material increase in calcium elimination in normal individuals or in cases of leprosy or exostosis, when magnesium in the diet was increased. Bogert and McKittrick (18) obtained a slight increase in urinary and fecal calcium of women to whom were given 6 gm. of magnesium lactate daily. Inspection of the data of Medes (19) revealed the finding that a high magnesium diet slightly depressed the calcium balance of rats. In a recent paper, Haag and Palmer (20), using growth as the index of changes in mineral metabolism, demonstrated with rats a deleterious effect of magnesium at some levels of calcium and phosphorus intake.

That ingested magnesium has some effect on calcium retention is scarcely to be doubted. Injection of magnesium salts certainly results in a loss of calcium, and oral intake might be expected to have a similar, though lessened, effect. Most of the evidence presented does, indeed, point toward a slight loss of calcium which can be ascribed to magnesium ingestion. Whether or not this is practically important is questionable. Sherman (21) states, "The 'ordinary mixed diet' of Americans and Europeans, at least among dwellers in cities and towns, is probably more often deficient in calcium than in any other chemical element." Under conditions of great calcium need, such as growth, pregnancy, and lactation, even a small loss might be of importance.

Likewise in rickets, the wide prevalence of which is well recognized (22), an interference in mineral metabolism might be of consequence. These practical considerations partially prompted the experiments which follow.

EXPERIMENTAL.

The purpose of these experiments was to study the effect of the ingestion of magnesium salts on the assimilation of calcium in the rat, using as criteria the ash content and mass of the bones and the growth in body weight. In later experiments the degree of calcification in the metaphyses was also determined by the examination of longitudinally cut bones stained with silver nitrate.

The technique has been varied necessarily as the work progressed in order that the problem might be attacked from different angles, but the same objective has been maintained throughout. The first feeding trials were concerned with the effect of magnesium salts under conditions of gross deficiency of calcium, as well as under conditions optimal with respect to calcium. In later trials

the effect of magnesium ingestion in the production of rickets on high calcium, low phosphorus diets was the subject of study.

Experiments with Low Calcium Basal Rations.

A. Effect of Addition of $MgCl_2$ in Amounts of 1 Gm. per 100 Gm. of Ration.—The purpose of the first series of feeding trials was to compare the effect of feeding magnesium salts at various levels of calcium intake. To this end magnesium chloride at a level of 1 gm. per 100 gm. of ration and calcium lactate at levels of 0 to 2 gm. were added to a synthetic basal ration. By this procedure any effect of magnesium at either low or high levels of calcium should be detectable.

The synthetic basal ration consisted of casein 18 parts, agar 2, yeast 6, cod liver oil 2, a salts mixture (Salts 45) 2.45, and dextrinized starch to 100 parts. Casein, used as the chief source of protein in the diet, was high grade commercial casein, which had been extracted for 5 days with a very dilute solution of hydrochloric acid (pH 4) in order to remove as much calcium as possible. The acid was changed twice a day and the casein washed with distilled water. The agar had been extracted in a similar manner for 6 days with 0.1 per cent hydrochloric acid, washed frequently for 3 days, dried, and ground. The yeast was ordinary commercial brewers' yeast which had been shown to be a suitable source of the vitamin B complex. The cod liver oil was added to the ration each week, since it had been found to become rancid if incorporated in the ration and exposed to the air for more than 1 week. The dextrin or dextrinized starch was ordinary corn-starch which had been moistened with water, autoclaved at 15 pounds steam pressure, dried, and ground.

The salt mixture employed (Salts 45) was composed of NaCl 0.73 gm., KCl 1.25, $MgSO_4$ (dry) 0.25, iron citrate 0.12, and KI 0.03. It was free from calcium and phosphorus. The latter element was omitted because of the action of soluble phosphates in counteracting the effects of magnesium salts, as demonstrated by the experiment of Hart and Steenbock (11) on swine. The inclusion of another variable would have complicated this problem, and the omission of additional phosphorus was permissible because the casein and other constituents of the basal ration supplied enough of this element for fair growth.

To this basal ration were added calcium lactate at levels of 0.0, 0.2, 0.4, 0.8, 1.6, and 3.2 gm. These rations were compared with similar ones which contained, in addition, 1 gm. of MgCl_2 per 100 gm. of ration, supplied in the form of a solution of known magnesium content, because of the very hygroscopic nature of the salt, which made it difficult to handle quantitatively in crystalline form.

Each of these twelve rations was fed to a group of four rats, whose ages at the beginning of the trial were 24 to 25 days and whose weights averaged 54 gm. The members of the litters were distributed in such a way as to compare the effect of MgCl_2 at all levels of calcium lactate within each litter. Each group was kept in a screened cage over screens. The rations and distilled water were fed *ad libitum*. Enough of each ration was made up each week to suffice for that time. Records were kept of the amounts of food consumed weekly; and although these were not strictly accurate because of some scattering of the ration by the rats, they made possible a good estimate of the food intake.

The rats were weighed each week and killed at the end of 11 weeks. The bones used for ash analysis were the femur and humerus. They were cleaned, fresh dried, extracted with 95 per cent alcohol and later with ether in a Soxhlet extractor, dried, and weighed. They were then ignited to a white ash and reweighed. From these data the percentage of ash was calculated.

The amounts of calcium and magnesium in the constituents of the basal ration were determined by McCrudden's method (23). The total calcium, calculated as CaO , in 100 gm. of basal ration was 0.062 gm. and the magnesium, calculated as MgO , was 0.025 gm. Considering that the average amount of feed eaten by each rat was 7 to 10 gm. daily, the calcium obtained from the basal ration was 0.004 to 0.006 gm. of CaO per rat per day and the magnesium not quite half that amount (0.0017 to 0.0025 gm. of MgO). This amount of calcium, though far from optimal, served to permit some growth, even on the basal ration.

Table I gives the results of this experiment in condensed form. The values given there represent averages for the groups of four rats each. Comparison with respect to ash content and weight of the bones, and growth in body weight of the rats receiving MgCl_2 in the ration with those which received no MgCl_2 , showed

that the feeding of $MgCl_2$ at a level of 1 gm. per 100 gm. of ration had no detrimental effect on bone development or growth of the animal either at low or high levels of calcium intake.

The ash content of the dried and extracted bones, which is the best index of bone development, and also the weight of the bones demonstrated the lack of any effect which might be attributed to magnesium. The averages compiled in Table I showed that the bones of the rats fed on the $MgCl_2$ -containing rations developed at least as well as those of the controls. The differences in growth,

TABLE I.

Effect of Addition of $MgCl_2$ to Rations of Varying Calcium Content on Bone Development and Growth of Rats.

Additions of Ca and Mg salts.		Ash content of bones.	Weight of bones.	Body weight of rats.			Weekly food consumption.
Calcium lactate.	$MgCl_2$			Initial.	Final.	Gain.	
gm. per 100 gm. ration	gm. per 100 gm. ration	per cent	gm.	gm.	gm.	gm.	gm.
None.	None.	35.4	0.098	51	127	76	187
	1.0	39.8	0.110	52	136	84	190
0.2	None.	43.7	0.131	52	141	89	215
	1.0	46.1	0.127	55	151	96	190
0.4	None.	48.4	0.131	54	165	111	237
	1.0	49.8	0.142	55	156	101	212
0.8	None.	54.8	0.174	52	188	136	240
	1.0	56.2	0.185	53	159	106	217
1.6	None.	60.9	0.273	52	216	164	287
	1.0	58.2	0.305	59	237	178	285
3.2	None.	60.7	0.228	51	183	132	253
	1.0	61.3	0.236	59	175	116	240

which were neither great nor constant, may be attributed, where found, to a lack of palatability of the $MgCl_2$ rations. This lack of palatability was demonstrated more directly by the records of food intake, which represented averages over the whole period of the experiment of the weekly consumption of the group.

The extreme deficiency in calcium of the basal ration was proved by the poor growth and bone development of the rats on this ration and by the great improvement in condition occasioned by calcium additions. The bones of the animals on the calcium-poor rations, although low in ash, were not typically rachitic, but rather

osteoporotic, being small and weak, but not swollen and distorted as in typical experimental rickets of the low phosphorus type.

The data here presented warrant the conclusion that rats fed on a ration which varied in calcium content from gross deficiency to an entire fulfillment of the calcium requirements, were not affected by the addition to the ration of relatively large amounts of magnesium chloride either in growth or in calcium assimilation, as shown by the weight and ash content of the bones.

B. Effect of Addition of Larger Amounts of $MgCl_2$.—In a second series of experiments an attempt was made to demonstrate any effects of feeding higher levels of $MgCl_2$. Since the addition of 1 gm. of $MgCl_2$ per 100 gm. of ration had not influenced growth or bone development, the amounts of this salt were increased to 2 and 4 gm. per 100 gm. of ration in addition to the 1 gm. level and controls receiving no $MgCl_2$. Calcium lactate was varied from 0 to 1.6 gm. The technique was similar to that employed in the previous trial.

The results of feeding $MgCl_2$ at high levels were inconclusive as regards any specific effect of magnesium on calcium assimilation because of other more serious disturbances occasioned by the ingestion of this salt. The most marked consequences were seen in the condition of the digestive tract. The rats which received rations containing 4 gm. per 100 of $MgCl_2$ failed to grow, and ten of the thirteen animals on the high magnesium rations declined in weight and died before the conclusion of the trial.

The first effects were extreme laxation, which resulted in the elimination of very soft, black feces. Later the intestines became atonic and fecal matter was retained in the cecum in a putrid green fluid mass. Autopsy revealed enormous distension of the cecum. In several cases the ceca were ligated, removed, and weighed with the contents. As a basis of comparison the stomachs of these animals were also weighed. The stomachs of normal rats were found to outweigh their ceca, but in the cases described the ceca with contents were 5 to 10 times as heavy as the stomachs. If the $MgCl_2$ in itself was not the toxic factor directly responsible for the death of these rats, the intestinal condition may have been the immediate cause.

The bones of these rats were weak and easily broken, and dark in color due to infiltration of blood. The weakness of bone was

shown externally by the huddled posture, waddling movements, and sensitiveness to touch. The ash content of the bones from rats receiving 4 gm. of $MgCl_2$ was, in general, lower than that of the controls and lower than the rats which received $MgCl_2$ at the 1 gm. and 2 gm. levels. However, a conclusion that this low ash content was due to any specific effect of magnesium on the calcium relations in the body is unwarranted, because of the toxicity of $MgCl_2$ and its most disturbing action on the digestive tract, which was sufficient to mask other effects.

The ingestion of $MgCl_2$ at lower levels than 4 gm. produced much less serious effects. A loss of intestinal tonus to a much smaller degree was observed in rats on the 2 gm. $MgCl_2$ level. The feces



FIG. 1. The effect of excessive amounts of $MgCl_2$. Rat 92 (left) was fed Ration 32, containing 1.6 gm. of calcium lactate and 4 gm. of $MgCl_2$; weight at start, 49 gm.; weight at end, 62 gm. Rat 85 (right) was fed Ration 31, the same in composition as Ration 32 except that it contained 1.6 gm. of calcium lactate and 2 gm. of $MgCl_2$; weight at start, 47 gm.; weight at end, 232 gm.

were black and loose and the intestines contained an accumulation of fecal matter somewhat greater than normal, but the general condition was much better.

To illustrate the effect of excessive amounts of $MgCl_2$ on growth and condition, a photograph is shown in Fig. 1 of two representative animals kept under the same conditions and fed rations exactly the same, except that the animal on the left received a ration to which had been added 4 gm. of $MgCl_2$ and the one on the right a ration containing 2 gm. of $MgCl_2$ per 100 gm. of ration.

The ash of the bones of rats on the 2 gm. level was very nearly equal to that of the controls, and that of rats on the 1 gm. level was fully equal to the controls. The latter finding corroborates the results obtained in the first experiment reported.

It is evident that amounts of $MgCl_2$ not so great as to produce profound digestive disturbances have little or no effect on growth

and bone development of rats under conditions either of calcium starvation or of an ample supply of calcium. Magnesium in large amounts must be fed in the form of a salt other than the chloride, if its further effect on calcium assimilation is to be studied.

C. Effect of Feeding $MgCO_3$.—In the third series of experiments $MgCO_3$ was employed as the source of the magnesium added to the ration. It was believed that the use of this salt would minimize the digestive disturbances which in the case of the chloride prohibited any conclusions as to the relations of calcium and magnesium when the latter was fed in large amounts.

A new basal ration, which had a calcium content even lower than the synthetic ration previously employed, was used. It consisted of yellow corn 77 parts, wheat gluten 20, cod liver oil 2, and sodium chloride 1. The calcium content of this ration was 0.045 gm. of CaO per 100 gm. as compared with 0.062 gm. per 100 gm. of the basal ration used in the previous trials. It was expected that the use of this ration would make the conditions increasingly exacting so that any possible effects of magnesium salts would be revealed.

The additions of both calcium and magnesium were made in the form of the carbonate, because it was believed that $MgCO_3$ would have much less effect upon the palatability of the ration and upon the alimentary tract than $MgCl_2$ and because the effects of the two elements would not be modified by the possibly different effects of the anions. Furthermore, data obtained with $MgCO_3$ might be of interest to those primarily concerned with the therapeutic effect of the hydroxide as milk of magnesia on calcium equilibrium.

Six rations were prepared: the basal ration alone and with additions of 0.5 gm. and 1 gm. of $MgCO_3$ per 100 gm. of ration, and the basal ration plus 0.5 gm. of $CaCO_3$ with additions of 1 gm. and 2 gm. of $MgCO_3$ per 100 gm. of basal ration. These were fed to groups of four rats, so distributed that each of the four litters of six rats had one representative on each ration.

The trial was allowed to run only 5 weeks in order that the greatest difference might be observed in the bone development; for continuation for a longer time might result in leveling out any differences resulting from the magnesium. For this reason the growth records showed only slight differences, none of which can be attributed to the presence of magnesium in the ration.

The analysis of the bones for ash content and weight comparisons, shown in Table II, disclosed no difference in either which could be ascribed to magnesium. The rations which contained no added calcium but varied in the MgCO_3 additions from 0 to 1 gm. per 100 gm. of ration, produced bones of nearly equal ash content namely, 38.6 per cent, as average for each group.

The addition of 0.5 gm. of CaCO_3 per 100 gm. of ration raised the ash content to about 52 per cent. No significant differences were produced by the addition of 1 or 2 gm. of MgCO_3 .

The food consumption records show that the rats consumed as much of the MgCO_3 -containing rations as of the controls. The figures represent in gm. the amount consumed per week by the group. They are averages for the whole period of the experiment.

TABLE II.
Effect of Addition of MgCO_3 to Rations of Varying Calcium Content.

Additions of Ca and Mg salts.		Ash content of bones.	Weight of bones.	Body weight of rats.			Weekly food consumption.
CaCO_3	MgCO_3			Initial.	Final.	Gain.	
gm. per 100 gm. ration	gm. per 100 gm. ration	per cent	gm.	gm.	gm.	gm.	gm.
None.	None.	38.5	0.0683	47	71	24	194
"	0.5	38.6	0.0723	46	81	35	190
"	1.0	38.7	0.0712	47	82	35	221
0.5	None.	52.7	0.1060	45	85	40	222
0.5	1.0	53.1	0.1130	45	100	55	273
0.5	2.0	51.8	0.1040	45	95	50	281

Although these records are not strictly comparable to those of previous series, in which MgCl_2 was added to the rations, because of different basal rations, they indicated that magnesium in the form of the carbonate is less distasteful than the chloride. Also 1 gm. of MgCO_3 contains about 12 per cent more magnesium than a gm. of MgCl_2 .

Thus the results obtained by adding magnesium in the form of the carbonate to the extent of 1 gm. per 100 gm. of ration to rations very deficient in calcium produced no effect on calcium assimilation, as shown by the growth or the ash content and weight of the bones of rats. The addition of 2 gm. of MgCO_3 per 100 gm. of ration to rations fortified with CaCO_3 likewise produced no effect.

Experiments with Rickets-Producing Rations.

A. Addition of $MgCO_3$ to Rickets-Producing Rations.—As related, no effect of ingested magnesium salts on growth or bone development was demonstrated by the previous technique in which diets very low in calcium as well as diets of optimum calcium content were employed, but in which an abundance of vitamin D was supplied in the cod liver oil. In the following experiments attempts were made to determine the effect of magnesium under conditions (a) of very severe rickets, (b) in the cure of rickets, and (c) in the presence of an ample amount of the antirachitic factor, vitamin D.

The rickets-producing ration was Ration 2965 of Steenbock and Black consisting of yellow corn 76 parts, wheat gluten 20, calcium carbonate 3, and sodium chloride 1 (24). This high calcium, low phosphorus ration produces very exaggerated experimental rickets in rats, characterized by extremely wide rachitic metaphyses, by great swelling of the ends of the long bones, by the production of large quantities of osteoid tissue, and by the absence of a provisional zone of calcification.

The low calcium rations used in the previous trials had produced bones low in ash, but without the gross changes found in low phosphorus rickets. The calcium-starved bone contained less osteoid tissue, and had a more nearly normal shape. The uncalcified areas in the metaphyses were not as wide. Low phosphorus rickets therefore appears to be a disturbance in skeletal metabolism even more profound than that due to lack of calcium. Under such rigorous conditions it was believed that any effect of magnesium would be made evident.

Park in his article in *Physiological Reviews* on rickets (25) states that the Johns Hopkins investigators produced severe rickets in rats by the addition to the diet of 1 to 4 per cent of $MgCO_3$. The rickets produced was of the low phosphorus type, and $MgCO_3$ was most effective in producing rickets when the phosphorus content of the ration was low. The higher levels of $MgCO_3$ caused the rats to die in a few weeks. No further data were given.

Although preliminary tests with the "recovery" method and the "line" test of McCollum and coworkers (26) failed to show any definite differences in calcium deposition between the bones of

rats which received 1 or 2 gm. of MgCO_3 per 100 gm. of ration and those which did not, nevertheless it was decided to make certain by a "prophylactic" test of longer duration whether or not MgCO_3 had any influence on calcification. The advantage of this latter type of experiment is that it gives definite comparative data expressed in per cent of ash in the bone.

MgCO_3 was therefore added to rachitogenic Ration 2965 in amounts of 0.5, 1.0, and 2.0 gm. per 100. Ration 2965 alone was fed as a control. Cod liver oil was varied in amount from 0 to 0.8 gm. per 100 gm. of ration. These rations were fed to groups of three rats for 4 weeks. Contrary to previous findings, in this experiment MgCO_3 produced a slight but definite diminution in ash of the bones at low levels of cod liver oil. This diminution in ash varied with the amount of MgCO_3 fed. 2.0 gm. of MgCO_3 added to 100 gm. of Ration 2965 reduced the ash from 34.6 per cent to 31.0 per cent. Similar slight reductions were observed at the 0.1, 0.2, and 0.4 gm. levels of cod liver oil but not at the 0.8 gm. level.

To verify this result, a more comprehensive experiment was performed with different CaCO_3 and MgCO_3 additions, both with and without cod liver oil. The first four rations were merely a repetition of the previous trial but with four rats in each group instead of three and an increased duration of the feeding trial, 5 weeks instead of 4. In addition consumption records were kept more accurately to test the possibility that unequal food intake might be a factor in the effect produced. The second portion of the experiment was designed to test the suggestion that increased basicity of the ration might be the cause of decreased calcification produced by the feeding of MgCO_3 . The studies of Zucker and his colleagues (27, 28) have shown that the ingestion of excess base may affect the inorganic metabolism of the body in the direction of the production of rickets. Alkalinity of the intestinal contents and feces has been found by many workers (29-32) to be increased in rickets and decreased by the addition of an antirachitic factor. Thus the possibility that an additive effect of CaCO_3 and MgCO_3 , increasing the alkalinity of ration and intestinal contents, might be responsible for the action of MgCO_3 . Therefore, in the fifth ration the 3 per cent of CaCO_3 was replaced by its equivalent in MgCO_3 , 2.53 gm. per 100 gm. of ration. In addition

to this ration, which was equivalent in basicity to Ration 2965, a sixth was prepared which contained 4.53 gm. of $MgCO_3$, equivalent in basicity to the fourth ration. These six rations were fed both without and with the addition of 0.8 gm. of cod liver oil per 100 gm. of ration, an amount previously found sufficient to prevent rickets.

TABLE III.

Effect of Addition of $MgCO_3$ to Rachitogenic and Non-Rachitogenic Rations.

CaCO ₃ in ration.	Addi- tions of MgCO ₃ .	Ash content of bones.	Weight of bones.	Body weight of rats.			Weekly food con- sumption	AgNO ₃ staining test *
				Initial.	Final.	Gain.		
No cod liver oil addition.								
per cent	gm per 100 gm. ration	per cent	gm.	gm.	gm.	gm.	gm.	
3	0.0	35.1	0.0906	62	101	39	225	— to +
3	0.5	32.1	0.0830	59	90	31	211	— to +
3	1.0	30.1	0.0812	61	89	28	208	—
3	2.0	31.0	0.0802	62	82	20	181	—
0	2.53	39.6	0.0739	59	68	9	168	++++
0	4.53	33.3	0.0664	57	69	12	161	++++
Addition of 0.8 gm. of cod liver oil per 100 gm. of ration.								
3	0.0	49.4	0.0973	56	83	27	210	++++
3	0.5	49.6	0.1008	57	84	27	226	++++
3	1.0	48.8	0.1090	60	87	27	226	++++
3	2.0	47.0	0.1007	59	84	25	219	++++
0	2.53	42.2	0.0908	63	98	35	227	++++
0	4.53	42.0	0.0823	62	84	22	186	++++

* Key to AgNO₃ staining test for calcification: — very wide rachitic metaphysis, + wide rachitic metaphysis, ++ medium rachitic metaphysis, +++ narrow rachitic metaphysis, ++++ complete calcification.

The litters of six rats each were so distributed in each series as to compare all six levels of $MgCO_3$ within the litters. In addition to the usual data, *i.e.* weight and ash content of bone and growth of rats, examination was made of the width of the metaphyses of radii and ulnæ when longitudinally split and stained with silver nitrate by exposure to light. The width of the uncalcified area was taken as indicative of the severity of the rickets.

The results shown in Table III are, as before, averages for the group. The percentage of ash in the bones and the silver nitrate staining test confirmed the results of the second trials, that the addition of MgCO_3 to the rachitogenic Ration 2965 increased the severity of the rickets directly as the amount of MgCO_3 was increased. The most pronounced effect of MgCO_3 was observed at low levels of cod liver oil. Even at the 0.8 gm. level a slight decrease in ash content was found in the ration containing 2 gm. of MgCO_3 . However, the abnormally low ash of one rat in this group of four lowered the average for the group. Thus any conclusion that the effect of MgCO_3 is revealed at high levels of cod liver oil as well as at low levels would be unwarranted.

The growth and the average weight of bone of the rats also follow the same trend as the percentage of ash and calcification as shown by the AgNO_3 staining test, in that they too decrease as the MgCO_3 in the diet increases. However, the fact that the food intake is decreased on high MgCO_3 rations makes interpretation of the results uncertain, since in order to establish definitely a specific effect of magnesium on calcium assimilation, the food intake should be equal on all rations. Variations in the amounts of ration consumed might produce changes in bone entirely apart from the mineral relations.

The series of experiments which was designed to test the possibility of a basicity effect, gave the following results. In rations containing no cod liver oil the percentage of ash of the bones of rats on 2.53 gm. of MgCO_3 per 100 gm. of ration was much greater than that of rats on Ration 2965 which contained 3 per cent of CaCO_3 , an amount equivalent in basicity to the 2.53 gm. of MgCO_3 . Similarly the level of 4.53 gm. of MgCO_3 also gave a percentage of ash higher than that of the fourth ration to which it corresponded in basicity.

The condition produced here was not rickets of the exaggerated type found in rats fed on the high calcium, low phosphorus Ration 2965. The bones of the animals on the high magnesium, low calcium rations were not typically rachitic. They were small, not swollen at the ends as are rachitic bones, and were weaker through the diaphyses than in the metaphyses. The tests for calcification by the AgNO_3 staining test showed practically no rachitic metaph-

yses in the radii and ulnæ of the rats on these rations. A deficiency of calcium affords an explanation for these results.

The food intake was also much less than normal. Especially toward the end of the trial did the consumption drop. The "starvation" effect in the deposition of bone salts, demonstrated by McCollum and his coworkers at Johns Hopkins (33) may also contribute toward the explanation of the absence of rachitic metaphyses. These investigators showed that fasting of rats, previously made rachitic, resulted in the deposition of calcium in the rachitic metaphyses.

The effect on the digestive system of excessive amounts of magnesium salts, first shown with high $MgCl_2$ rations, was observed also in this series, especially on levels of 2.53 and 4.53 gm. of $MgCO_3$. The loss of tonus of the intestine resulted in a retention of fecal matter in the ceca. This produced a marked distension of the abdomen. The accumulation of feces also produced an apparent weight much higher than the actual body weight; therefore the growth records do not tell the whole truth. In rats on Ration 2965 with added $MgCO_3$ the condition of the alimentary tract was normal, except that some animals on the 2 gm. of $MgCO_3$ ration showed a slight loss of tonus of the tract. So growth on Ration 2965 with and without additions of $MgCO_3$ was considerably greater than that on the low calcium rations.

The addition of cod liver oil to the rations in which the $CaCO_3$ was omitted did not raise the ash or weight of the bones to the degree observed in rations in which $CaCO_3$ was in excess. It is evident that the lack of calcium in this ration was the limiting factor. Our experiments on low calcium diets demonstrated that even the presence of 2 per cent of cod liver oil in the ration was unable to raise the ash to normal when calcium was grossly deficient.

B. Comparison of Effects of Ingestion of Equal Amounts of Magnesium in the Form of Chloride and Carbonate.—These experiments consisted of a series of trials designed to eliminate the possibility that unequal food intake was a factor in the observed detrimental effect of $MgCO_3$ on bone development. Instead of feeding the rations *ad libitum* as had been done in all previous trials, an equal amount of ration was given each rat. Each animal was isolated in a separate cage. The ration was weighed out daily,

and no more was given any rat than the amount taken by the rat consuming the least. The chief disadvantage of this method was that it limited the consumption of all to the level of the poorest animals and most unpalatable rations, and consequently retarded the growth of all. Actually the average daily food intake was only 4.25 gm. for the 5 week period, and it fell to below 4 gm. during the last 2 weeks of the experiment.

The experiment was also so designed as to test again the hypothesis of basicity as an explanation for the MgCO_3 effect. Ration

TABLE IV.
Comparison of Effect of MgCO_3 and MgCl_2 Added in Equivalent Amounts

Additions of magnesium salts.	Ash content of bones.	Weight of bones.	Body weight of rats.			Length of bones	AgNO ₃ staining test.
			Initial.	Final	Gain		
No cod liver oil additions.							
<i>gm. per 100 gm. ration</i>	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>mm.</i>	
None (Ration 2965).	34.5	0.0647	51	57	6	19.0	+
1 gm. MgCO ₃ .	32.8	0.0564	48	51	3	18.4	+
MgCl ₂ equivalent in Mg to 1 gm. MgCO ₃ .	36.4	0.0574	48	49	1	18.6	++
Addition of 2.0 gm. of cod liver oil per 100 gm. of ration.							
None.	49.4	0.0755	49	57	8	20.2	++++
1 gm. MgCO ₃ .	47.9	0.0689	48	51	3	19.6	++++
MgCl ₂ equivalent in Mg to 1 gm. MgCO ₃ .	47.0	0.0647	49	49	0	19.2	++++

2965 alone was used as a control. MgCO_3 was added to Ration 2965 at a level of 1 gm. per 100 gm. of ration. MgCl_2 was added to Ration 2965 in an amount equivalent in magnesium to 1 gm. of MgCO_3 . The cod liver oil levels were 0 and 2 gm. The MgCO_3 was added to the rations in the form of a powder. The MgCl_2 was added as an aqueous solution standardized with respect to its magnesium content, and was evaporated on the basal ration.

The data obtained include, in addition to the ash content and weight of bone, the silver nitrate staining test for calcification, and growth records, also the average lengths of the femora and the humeri (Table IV).

Consideration only of certain of the criteria of calcification, namely the percentage of ash and the silver nitrate staining test, would lead to the definite conclusion that basicity is the cause of the impairment of calcification produced by the ingestion of MgCO_3 , since the percentage of ash and the appearance of the metaphyses of the bones of rats on the MgCl_2 ration showed that better calcification was induced by feeding of this ration than by the feeding of the basal Ration 2965 or the MgCO_3 ration. This was true under the rachitic conditions obtaining in the groups receiving no cod liver oil. In the non-rachitic groups receiving 2 gm. of cod liver oil per 100 gm. of ration the magnesium additions produced only a slight diminution in ash, which was the same for the chloride and the carbonate.

However, other factors must be considered before ascribing these effects solely to basicity. First, inspection of the growth records shows that the growth of rats on all rations was very poor, very far from normal. Yet even these records show poorer growth on the magnesium-containing rations than on the basal ration. Rations containing MgCl_2 equivalent in magnesium content to 1 gm. of MgCO_3 produced no increase in weight. Table IV also gives averages of the length of the femora and humeri, an index of growth which supplements the growth records or measures of body weight. The results of these measurements corroborate the growth records in showing that growth was depressed on the magnesium-containing rations.

Growth, not only in body weight and bone length, but also in weight of bone, was depressed by the magnesium salts. The depression was very nearly equal for the chloride and carbonate, and the difference found in the percentage of ash was not observed in the weight of bone. The explanation for the poor growth in general lies in the failure to consume the rations, which in turn rests partly with the age and size of the rats at the start of the trial. The ages were only 21 to 23 days and the weights averaged less than 49 gm. The animals were too young to consume the rather unpalatable rations.

C. Lack of Any Effect of MgCO_3 or MgCl_2 under Conditions of Extreme Rickets.—In this trial the factors of inequality of food intake and lack of normal food consumption were largely eliminated. Both of these difficulties were solved partly by moistening

the daily ration with water, a procedure which reduced scattering of the ration to a minimum and also seemed to increase palatability of the ration, and partly by the use of rats of greater age and weight than those used in the previous trial.

The rations, made up each day and fed in equal amounts to rats in individual cages, were moistened with a volume of water amounting to one-fifth of the weight of the dry ration. The consistency of the ration was good, moist but not dough-like. It was more palatable than the dry powdery ration previously employed and less was scattered.

The rats ranged in age from 25 to 28 days and in weight from 50 to 62 gm., averaging 58 gm. Animals of this age and size

TABLE V.

Lack of Any Effect of $MgCO_3$ or $MgCl_2$ under Conditions of Extreme Rickets.

Additions of magnesium salts.	Ash content of bones.	Weight of bones.	Body weight of rats.			AgNO ₃ stain- ing test.
			Initial.	Final.	Gain.	
<i>gm. per 100 gm. ration</i>	<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
None (Ration 2965).	30.2	0.0771	58	76	18	—
1.0 gm. $MgCO_3$.	29.7	0.0770	57	73	16	—
$MgCl_2$ equivalent in Mg to 0.5 gm. $MgCO_3$.	30.3	0.0739	56	72	16	—
$MgCl_2$ equivalent in Mg to 1.0 gm. $MgCO_3$.	30.8	0.0722	58	70	12	—

are able to cope with deficient rations better than are the younger and smaller ones. Six animals comprised a group, in order to obtain sufficient data to make the averages more reliable.

The rations were the same as those in the previous series, with the exception that an additional level of $MgCl_2$ was fed. Only the rickets-producing rations were used; the cod liver oil was eliminated.

The results of this experiment are clearly shown in the tables of ash content and weight of bones (Table V). The animals were very rachitic and no differences were obtained by the addition to Ration 2965 of either the chloride or carbonate of magnesium. The silver nitrate staining tests showed wide rachitic metaphyses in the radii and ulnæ of all the animals in the series. The ends of the bones were swollen. The picture presented was one of very severe rickets in all cases.

The food consumption was greatly increased over that obtained in the previous trial. An intake of 6 gm. per day was maintained by all animals throughout the 5 week period except that during the last few days small amounts of ration were left by some of the rats on the magnesium rations. The MgCl_2 rations limited the food intake of all. The increased consumption resulted in improved growth, which made possible the development of more pronounced rickets than is obtainable under conditions of partial fasting, such as prevailed before. Only slight differences in growth in body weight and length were found between rats on the basal ration and the magnesium-containing rations and between the rats on the different forms of magnesium. A slight loss of intestinal tonus, shown by accumulation of fecal matter in the ceca, was observed in rats receiving MgCl_2 equivalent to 1 gm. of MgCO_3 ; but the condition did not approach in severity that observed in previous trials with larger amounts of magnesium salts.

Thus the addition of MgCO_3 or MgCl_2 to a ration containing 3 per cent of CaCO_3 did not increase the severity of the rickets, when food intake was equalized and increased.

DISCUSSION.

The failure of these experiments to show a definite additive effect of the carbonates of calcium and magnesium as a depressant of bone salt deposition was unexpected in view of the chemical similarity of these elements, particularly as regards solubility of their phosphates in an alkaline medium. The possibility is not excluded that, in less complete rickets than were produced in the last series of experiments, such an effect may obtain. The rickets produced in that trial was of a very severe type—the ash was very low, the rachitic metaphyses very wide, and the joints very swollen. Under these conditions of maximum rickets, the added base in the form of MgCO_3 exerted no effect.

In this connection the observations of Haldane (34) and of Gamble, Ross, and Tisdall (35) that CaCl_2 , though a chemically neutral salt, may nevertheless act as an acid salt in the metabolism of the animal, presumably with increase in basicity of the intestinal contents, are of interest. Haldane's later observation (36) that MgCl_2 has a similar action may explain the failure of MgCl_2 to produce an effect attributable to a reduced alkalinity of the tract,

since the ultimate effect in the intestines appears to be that of a base.

Recent experiments in this laboratory (37) bear upon the possibility of basicity as a factor in calcium and magnesium relations. MgCO_3 added to a ration deficient in phosphorus and vitamin D and containing CaCO_3 in only moderate amounts, caused a reduction of the inorganic phosphorus of the blood serum and a slightly lowered percentage of ash in the bones of rats. However, these effects of MgCO_3 were much less pronounced than those of an equivalent amount of CaCO_3 .

The studies reported in this paper have demonstrated that the ingestion of large amounts of magnesium salts produces physiological disturbances, in rats, serious enough to mask any effects of mineral antagonisms. The action on the digestive tract, first demonstrated with MgCl_2 and later, to a lesser extent, with MgCO_3 , precludes feeding these salts in large amounts. Further experiments in the direction of increasing the intake of magnesium salts, we deem unprofitable.

Park (25), in his statement that MgCO_3 produces rickets of great severity, entirely disregards the other effects of magnesium so clearly brought out here. The very fact that his animals died after being fed on high magnesium diets suggests that rickets, which rarely kills, was complicated with other disorders, undoubtedly digestive, which were more serious physiologically than rickets.

Our experiments have failed to show any effect of magnesium salts which could not be attributed to effects other than a specific one on calcium assimilation. No evidence has been presented that, under the most drastic conditions of disturbance in calcium metabolism, magnesium has a deleterious effect on calcium assimilation in the rat. Additions of magnesium salts to diets grossly deficient in calcium produced no change in the bones. Normal diets supplemented with calcium and cod liver oil were not affected by magnesium additions. In healing rickets magnesium did not retard calcium deposition in the metaphyses; nor added to a rickets-producing ration did it increase the severity of the rickets when conditions of food intake and growth were equalized.

Quite similar findings have been reported by Hart, Steenbock, and Morrison (38) in their studies upon dolomite as a lime supple-

ment for domestic animals. Dolomite containing 40 per cent of MgCO_3 produced normal growth and reproduction in cattle, swine, and chicks. Excessive amounts of pure MgCO_3 (8 per cent of the ration) proved disastrous for chicks, resulting in 70 per cent mortality and very poor growth and bone development.

Consideration of calcium magnesium relations from a practical standpoint, as revealed by our rat experiments, forces the conclusion that magnesium has no demonstrable effect on calcium retention in the body under ordinary conditions of diet or administration of magnesium salts, since normally the conditions would never approach in severity those produced in these experiments.

CONCLUSIONS.

Excessive amounts of magnesium salts, introduced into the rations of rats, resulted in such reduced palatability and lowered food intake, accompanied by such severe digestive disturbances, that any specific effect of magnesium on calcium relations in the body was masked.

When lesser amounts of magnesium were fed, amounts which, however, were still relatively large, such disturbances were not manifested. Under these conditions of administration, no effect on calcium assimilation was demonstrable by additions of magnesium salts in the form of either chloride or carbonate to rations grossly deficient in calcium or supplemented with adequate amounts of calcium. Likewise in the prevention, production, and cure of rickets, in which high calcium, low phosphorus rations were employed, additions of MgCl_2 or MgCO_3 did not increase the severity of the rickets, when the food intake was controlled.

The lack of any deleterious action of magnesium salts under the rigorous conditions described here minimizes the probability of danger in the ordinary therapeutic use of magnesium salts. The digestive tract in its selective absorptive capacity apparently represents an excellent protective mechanism for the exclusion of an excess of magnesium.

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FURTHER PROGRESS TOWARDS THE ISOLATION OF THE ANTINEURITIC VITAMIN (VITAMIN B) FROM BREWERS' YEAST.

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The antineuritic concentrate prepared by the moderately large scale method described¹ 3 years ago has, until recently, resisted all efforts to convert it into a more active fraction. An observation made while benzoylating several samples, preparatory to testing them by the Pauly reaction, furnished a clue which has led to a process for eliminating a considerable proportion of the inactive nitrogenous constituents contained in the concentrate. This consists in adding an excess of benzoyl chloride to an aqueous solution of the concentrate, made alkaline with sodium carbonate, and extracting the mixture with chloroform. The aqueous solution remaining after the extraction contains a large amount of sodium chloride and other salts, but only about one-fourth of the nitrogen originally present. When tested on pigeons it is found to possess an activity corresponding to the major part of the vitamin of the concentrate from which it was prepared. Hence, benzoylation in alkaline solution and extraction with chloroform effects a removal of inactive nitrogenous constituents without seriously damaging the antineuritic compound.

It has also been found that when this highly active aqueous solution is poured into about 10 volumes of acetone the salt mixture which is precipitated contains less than one-half of the nitrogen but practically all of the antineuritic vitamin originally present. This carrying down of the active material by the inorganic salts was quite unexpected. It calls attention to a property of the antineuritic vitamin which may be utilized advantageously in

¹ Seidell, A., *J. Biol. Chem.*, **67**, 593 (1926).

procedures for its isolation. The final product made in this way is a nearly white mixture of salts containing usually about 0.25 per cent of nitrogen. It protects pigeons from loss in weight on a diet of polished rice in doses of about 60 mg.

On the assumption that the antineuritic vitamin is a nitrogenous compound, the extent of the purification so far effected is shown by the following comparison of the mg. of nitrogen at the several stages of the procedure required to protect pigeons on a diet of polished rice.

Fraction tested.	N present.	Protective dose.	N in protective dose.
	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>
Dried brewers' yeast.....	9.4	200	18.8
Activated solid.....	2.0	150	3.0
Vitamin concentrate (1926).....	6.5	23	1.5
Aqueous solution after benzylation.....			0.4
Vitamin salts precipitated by acetone.....	0.25	60	0.15

EXPERIMENTAL.

Preparation of Vitamin Concentrate.—The process for preparing a stable, dry antineuritic concentrate described 3 years ago has not been modified essentially since that time. There is one observation, however, to which attention should be called. The use of acetic acid for neutralizing the aqueous sodium hydroxide extract of "activated solid" may result in a destruction of vitamin due to alkalinity which develops if the concentration of the extract by vacuum distillation is pushed too far. It is therefore better to use sulfuric acid in all cases. A too great excess of this acid may be avoided by titrating and adjusting the reaction to such a hydrogen ion concentration that after distillation the pH of the solution will be about 3.0.

It should also be mentioned that in the steps required for removing the excess of $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ and insoluble organic impurities the addition of alcohol should not be continued much beyond 50 per cent. A higher concentration causes the precipitation of a portion of the vitamin with the impurities which are thrown out. There will, of course, be a larger amount of salts and extraneous materials left in the final dehydrated residue from the 50 per cent

alcoholic solution, but the dry concentrate will contain a larger proportion of the vitamin originally present in the yeast.

The vitamin concentrate used for the experiments to be described later was made as follows: 3000 gm. of "activated solid" were mixed with 26 liters of distilled water, and 4 liters of water containing 600 gm. of sodium hydroxide were added. The mixture was violently agitated for 4 minutes and then put through a Sharples supercentrifuge in the next 2 to 4 minutes. The nearly clear effluent was received in a vessel to which 900 cc. of a mixture of 1 volume of concentrated H_2SO_4 and 2 volumes of water were immediately added. The foam was discharged by addition of about 1 liter of alcohol. The reaction of the solution was adjusted by titration so that 25 cc. required 0.3 cc. of N NaOH , methyl red being used as indicator.

Another 3000 gm. of "activated solid" were treated exactly as above and the resulting solution mixed with that obtained from the first 3000 gm. of "activated solid." The approximately 60 liters of aqueous extract containing some suspended flocculent material were distilled under diminished pressure to approximately 15 liters. This solution was allowed to stand at about 4° for 3 days. Approximately 12,500 cc. of clear solution were drained from the deposit of $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ and organic material which separated and 4000 cc. of 95 per cent ethyl alcohol were added. This mixture was kept another day at 4° . An additional amount of $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ and organic matter separated and was removed by filtration on a Buchner funnel. The filtrate amounted to 14,800 cc. and on titration 5.0 cc. required 2.2 cc. of 0.1 N NaOH solution, methyl red being used as indicator. Its pH was 2.8 to 3.0. An additional 4000 cc. of 95 per cent ethyl alcohol were added and after standing 2 days at 4° a further amount of $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ and organic matter separated. This was removed by filtration and the final clear solution was divided into four portions of about 4500 cc. each. These were separately distilled under diminished pressure to near dryness and the residues treated with absolute ethyl alcohol repeatedly until converted to an anhydrous condition. Each portion yielded approximately 100 gm. of a dry, slightly yellowish powder. This vitamin concentrate which was designated Sample 26.164 was found to contain approximately 35 per cent of ash, weighed after igniting with an excess of sulfuric acid.

Its nitrogen content was 6.49 per cent. Its activity as determined by administration on alternate days to pigeons fed exclusively on polished rice is shown in Table I.

It is evident that the minimum amount of Sample 26.164 required to protect pigeons is slightly more than 20 mg., containing approximately 1.5 mg. of nitrogen.

Benzoylation.—The concentrate, Sample 26.164, was benzoylated by dissolving about 20 gm. of it in 100 cc. of water, clarifying the solution by centrifugation and decantation, adding 25 gm. of anhydrous sodium carbonate, and then 40 cc. of benzoyl chloride. Abundant evolution of carbon dioxide occurs and the mixture be-

TABLE I.
Activity of Vitamin Concentrate, Sample 26.164, on Pigeons.

Date.	Dose given.	N present in dose given.	No. of pigeons used.	Duration of test.	Average change in weight of each pigeon.
	mg.	mg.		days	gm.
Oct. 3-13	10	0.65	5	10	-17
" 3-13	10	0.65	4	10	-13.5
Jan. 13-23	18	1.17	4	10	-2
Oct. 3-13	20	1.30	5	10	-1
" 3-13	20	1.30	5	10	-5.5
Jan. 13-23	23	1.50	5	10	+15
Oct. 3-13	30	1.95	3	10	+1
" 7-19	30	1.95	4	12	+14
" 13-23	30	1.95	5	10	+17
Nov. 6-16	30	1.95	10	10	+22.3

comes warm. In order to prevent loss by foaming the reaction should be conducted in a large beaker.

Extraction.—The mixture is extracted by stirring vigorously with about 200 cc. of chloroform, and separating the two layers by centrifugation. The lower chloroform extract is conveniently withdrawn by means of a pipette. The aqueous layer, which contains insoluble material, is then diluted with about an equal volume of water and brought to distinct acidity to litmus paper by repeated additions of small amounts of concentrated hydrochloric acid. The acidified mixture is then extracted as before successively with 200, 150, 100, and 50 cc. of chloroform. The insoluble

material is removed by centrifugation and filtration and the aqueous solution diluted to a convenient volume. The pH of this solution is usually about 3.0.

The nitrogen distribution and activity of the several fractions obtained in a typical experiment were as follows: With 20.0 gm. of concentrate, Sample 26.164, (containing 1.3 gm. of N) there were obtained:

1	Sample 27.158, 680 cc CHCl ₃ extract containing	0.751 gm. N		
2.	" 27.159, 2.77 gm CHCl ₃ and H ₂ O, insoluble precipitate containing	0.239	"	"
3	Sample 27.160, 500 cc aqueous solution containing	0.327	"	"
	Total	1.317	"	"

TABLE II

Results of Physiological Tests with Various Fractions of Vitamin Concentrate

Sample No	N contained in dose given	No. of pigeons used	Duration of test	Average change in weight of each pigeon
	mg		days	gm
27.158	3.6	5	4	-20
	7.2	2	4	-16
27.159	3.0	4	8	-2
	6.9	4	8	+9
27.160	0.62	5	8	+9
	0.50	5	8	+10
	0.38	4	10	-2

The physiological tests² of these fractions made on pigeons gave the results presented in Table II.

On calculation from these results and the activity of Sample 26.164 as previously determined (Table I) it is found that of the approximately $(20.0 \div 0.023 =)$ 870 protective doses of antineuritic vitamin in the 20 gm. of concentrate used for the experiment, the chloroform extract contained practically none, the insoluble precipitate only about $(0.239 \div 0.0035 =)$ 70 units, and the final aqueous solution approximately $(0.320 \div 0.0004 =)$ 800 protective doses. Thus about 90 per cent of the total activity remains

² Seidell, A., *Pub. Health Rep., U. S. P. H. S.*, **37**, 1519 (1922).

associated with the about 25 per cent of the original nitrogen which is present in the aqueous solution remaining after benzylation and extraction with chloroform.

Acetone Precipitation.—Preliminary attempts were made to remove a portion, at least, of the salts from the above active aqueous solution by evaporating to near dryness and extracting with various concentrations of ethyl and of methyl alcohol. It was found that in all cases the alcohol-insoluble salts thus eliminated retained a large proportion of the active material. This was especially the case when alcohol of higher concentration was used for the extraction. It was apparent that the active material contained in the salt mixtures was very slightly, if at all, soluble in pure ethyl alcohol.

Attention was then directed to the use of acetone for separating the inactive from the active constituents of the aqueous solution. The experiments were made simply by pouring the aqueous solution into enough acetone to cause the precipitation of a greater or lesser proportion of the salts. On testing the precipitates thus obtained it was found that they contained from 75 to 90 per cent of the active material, but usually less than half of the nitrogen. Direct tests of the activity of the acetone solution showed that no protection was afforded even by doses containing amounts of nitrogen in excess of that contained in protective doses of the original concentrate.

Of the several experiments which were made, a typical one was as follows: 200 cc. of an aqueous solution remaining after benzylation, containing 0.1414 gm. of N, corresponding to approximately 430 protective units of vitamin, were poured into 2500 cc. of acetone and the mixture was well shaken. The precipitated salts weighed 21.2 gm. and contained 0.3 per cent of nitrogen. The acetone solution measured 2710 cc. and contained 0.0791 gm. of nitrogen. The physiological tests of these two samples gave the results presented in Table III.

From this experiment it is evident that the 21.2 gm. of precipitated salts afford protection in doses of 0.050 to 0.060 gm. Therefore $21.2 \div 0.055 = 385$ protective doses. Since the estimated number present was 430, the actual recovery of antineuritic material was approximately 90 per cent.

Other experiments showed that when the precipitation was

made with less acetone, for example 8 or 9 volumes to 1 of the aqueous solution instead of 12.5, only about 75 per cent of the active material was recovered with the precipitated salts.

Physiological tests of several of the samples of vitamin-containing salts gave the results presented in Table IV.

TABLE III.
Physiological Tests of Two Samples of Precipitated Salts.

Sample No.	Dose given.	N present in dose given.	No. of pigeons used.	Duration of test.	Average change in weight of each pigeon.
	<i>gm.</i>	<i>mg.</i>		<i>days</i>	<i>gm.</i>
Precipitated salts.					
27.152	0.075	0.225	5	8	+9
	0.060	0.18	5	8	+3
	0.050	0.15	5	8	-2½
Acetone solution.					
27.153		2.22	3	6	-21

TABLE IV.
Physiological Tests of Samples of Vitamin-Containing Salts.

Sample No.	N in sample.	Dose given.	N present in dose given.	No. of pigeons used.	Duration of test.	Average change in weight of each pigeon.
	<i>per cent</i>	<i>mg.</i>	<i>mg.</i>		<i>days</i>	<i>gm.</i>
27.131 A	0.174	90	0.157	5	8	+3
27.139	0.257	75	0.193	5	8	+9
27.139	0.257	50	0.128	5	8	-4
27.145	0.399	50	0.20	5	8	+10
27.145	0.399	40	0.16	4	8	±0
27.145	0.399	30	0.12	5	8	-6
27.146	0.107	150	0.15	3	8	±0
27.152	0.30	75	0.225	5	8	+9
27.152	0.30	60	0.18	5	8	+3
27.152	0.30	50	0.15	5	8	-2½

In each case the minimum dose which prevented loss in weight was that containing approximately 0.15 mg. of nitrogen. The quantity of each sample required to protect varied inversely with the percentage of nitrogen which it contained. Hence, it appears most probable that the activity resides in the nitrogenous compound which is present.

Although the evidence so far obtained in regard to the composition of the antineuritic vitamin indicates that it contains nitrogen, there is as yet no indisputable proof of this point. It is true that the physiologically active compounds which have been isolated all contain nitrogen but in no case has a sufficient quantity been obtained to permit of the rigorous purification necessary to insure the absence of minute non-nitrogenous impurities which might have been responsible for the observed activity. The present results simply furnish additional evidence in support of the view that the antineuritic vitamin is a nitrogenous compound.

Since the antineuritic constituent of the above described salt mixture evidently possesses solubility characteristics resembling closely those of the salts present, a very careful study will be necessary to develop a method of separating the active from the inactive components of the mixture. In the meantime the procedure here described may be regarded as another step on the route toward the isolation of the antineuritic vitamin (vitamin B).

SUMMARY.

A further purification of the antineuritic concentrate previously prepared has been effected by benzoylation in alkaline solution and extracting with chloroform. The aqueous solution remaining after extraction, when poured into 10 volumes of acetone, yields a precipitate of salts accompanied by a nitrogenous compound which protects pigeons from loss in weight on a diet of polished rice in doses containing 0.15 mg. of nitrogen. Judged on the basis of nitrogen activity this final product represents a purification of antineuritic material more than 100 times that of dried brewers' yeast.

A CHEMICAL STUDY OF TYPE III PNEUMOCOCCI.*

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In 1917, in a study of the pneumococci, Dochez and Avery (1) showed that there was present in the fluid media of pneumococcus cultures a substance which precipitated specifically in antipneumococcus serum of homologous type. Further work by Heidelberger and Avery (2-4) has shown that two specific substances could be isolated from Types I, II, and III pneumococci; namely, a species specific protein and a type specific polysaccharide. The specific polysaccharide was different for each type of pneumococci and gave specific precipitation in high dilution with the homologous antipneumococcus serum. Avery and Heidelberger (5) made a study of the nucleoprotein material precipitated by dilute acid from solutions of Type II pneumococci in bile and found it had the characteristics of nucleoprotein and mucoid.

In this chemical study of Type III pneumococci, an effort has been made to follow the technique recommended by Johnson (6) and others (7-12) in their chemical study of the tubercle bacillus, with such modifications as were necessary to adapt it to the pneumococcus.

Growth of Pneumococci.

The virulent Type III pneumococci were grown in the following clear beef infusion broth (13) and separated from the medium after 16 to 18 hours incubation at 37.5° by a supercentrifuge.

	gm.
Beef, chopped.....	500
Distilled water.....	1000
Peptone.....	10
Sodium chloride.....	5
Glucose.....	0.2

* An abstract of this paper was presented at the meeting of the Division of Biological Chemistry of the American Chemical Society, Swampscott, Massachusetts, September 12, 1928.

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The organisms collected from 10 to 20 liters of medium were washed while in the centrifuge bowl by sending a liter of distilled water through the centrifuge. They were scraped from the bowl and dried at 55° in a thin layer, the drying requiring about 5 hours. The material formed thin, transparent, brittle, pale yellow pieces, and was ground to powder and dried to constant weight at 55°. 10 to 15 liters of medium yielded about 1 gm. of material.

Isolation of Soluble Specific Substance from Culture Medium.

Avery and Heidelberger (3, 4) had isolated a soluble specific carbohydrate from 7 day autolyzed mass culture of Type III pneumococci grown in phosphate-buffered broth. In order to see if such a substance could be isolated from an unbuffered culture medium from which the organisms had been separated by a super-centrifuge after 18 hours growth, the concentrated medium was examined according to their method. About 2 gm. of such material were isolated from 200 liters of medium. It corresponded in all respects to the material described by Heidelberger and Goebel (14, 15).

As a control, 80 liters of uninoculated medium were concentrated and examined by the same method (3, 4) in an attempt to isolate some similar material. None was found. It showed that the specific substance was either a metabolic or a disintegration product of the pneumococci. This is added evidence to that brought out by Avery and Heidelberger, that these polysaccharides are the specific substance and that they do not represent inert material carrying an extremely minute amount of active material.

Chemical Analysis of Pneumococci.

Analysis of Pneumococci Dried at 55°.

	<i>per cent</i>
Volatile matter at 105°.....	4.77*
Ash	8.64, 8.48*
Nitrogen (Kjeldahl).....	13.02, 13.32, 13.00*
" (Dumas).....	13.07*
" (Van Slyke).....	.
Acid-insoluble humin N.....	0.2
Acid-soluble " 	0.2
Ammonia N.....	1.0

* Analyses obtained by microchemical methods of Fritz Pregl.

TABLE I.
Extraction of Type III Pneumococci Dried to Constant Weight at 55°.
Analysis of Fractions Isolated.

Sample No.	Material analyzed.	Weight.	Initial material.	Loss at 105°.	Ash.	N.	P.
		gm.	per cent	per cent	per cent	per cent	per cent
1	Dried pneumococci (initial material).	39.441		4.77	8.48	13.00	2.94
2	Ether-soluble extract.	0.2938	0.74		0.20	0.00	Trace
4	Acetone-soluble "	0.1275	0.32		0.91	3.10	0.38
5	Absolute alcohol "	0.7832	1.98	3.75	9.20	6.43	1.06
6	Benzene extract.	0.00				6.50	1.10
7	Residue after extraction with ether, acetone, alcohol, and benzene.					12.84	2.25
8	Water extract.		60.4 (calculated).	3.40	8.88	12.80	2.36
9	Residue after water extraction.					14.42	0.90
10	Material precipitated by dilute acetic acid from water extract.			2.55	3.65	14.39	0.96
11	Soluble specific substance precipitated from water extract			3.47	1.74	15.55	3.43
14	Residue after 0.1 N sodium carbonate extraction of Sample 9		0.63	0.00	0.00	0.00	0.00
15	Material precipitated by dilute acetic acid from 0.1 N sodium carbonate extract.		4.4	4.23	5.16	14.28	2.07
16	Material precipitated by dilute acetic acid from 0.1 N sodium hydroxide extract.		21.1	4.53	1.42	14.88	0.33
17	Residue after 0.1 N sodium hydroxide extraction.			4.04	13.32	10.95	0.45

* These analytical results were obtained by use of the microanalytical methods of Fritz Pregl. Minute quantities of less than 5 mg were used in these analyses except in the two phosphorus determinations of the dried pneumococci.

TABLE I—*Concluded.*

Sample No.	Material analyzed.	Weight.	Initial material.	Loss at 105°.	Ash.	N.	P.
		gm.	per cent	per cent	per cent	per cent	per cent
18	0.1 N acetic acid extract.						
19	0.1 " hydrochloric acid extract.		0.64	3.70	9.42	10.46 10.36	0.59 0.44
20	Final residue.			1.84	3.76	12.69 12.81	0.26 0.27

Total N of bases.....	5.5
" " in filtrate from bases.....	8.6
Phosphorus.....	2.92, 2.94
Chlorine.....	0.71
Sulfur.....	0.32

The dried pneumococci gave positive biuret, Millon's, xanthoproteic, Molisch, Ehrlich's diazo, and *p*-dimethylaminobenzaldehyde reactions. The test for loosely bound sulfur was negative, no brown or black color developing after boiling with lead acetate and 30 per cent sodium hydroxide. No evidence of unoxidized or loosely bound sulfur could be obtained with any of the pneumococcus fractions, and in the nitroprusside test for sulfur a considerable quantity had to be taken in the sodium fusion to give a positive reaction. It seemed very probable that during the drying of the pneumococci the loosely bound sulfur became oxidized, as a sample of pneumococci dried on removal from the centrifuge by immediate immersion in a 50 per cent mixture of alcohol and ether saturated with carbon dioxide, gave a positive loosely bound sulfur test.

Extraction of Pneumococci.

The dry pneumococci were extracted with dry ether by placing them in a bottle and allowing successive 400 to 500 cc. lots of ether to stand on the material with occasional shaking at room temperature for 4 or 5 days. The clear ether was drawn off and filtered to remove suspended particles. When further portions of

ether removed no more material, the combined ether extracts were distilled and the residue was dried to constant weight at 55°.

After ether, the extraction was repeated with acetone, absolute ethyl alcohol, and benzene in the order named, the extracts being dried at 55°. Benzene removed no material. The percentages extracted were calculated on the original weight of the pneumococci, deductions being made for samples of material removed for analysis. (See Table I.) The numbers of the materials in the text correspond to the numbers in Tables I and II.

The ether extract (Sample 2) was a yellow, fatty material, which was solid at room temperature and melted at 37–40°. On ignition it distilled, leaving no ash. It contained no nitrogen and only a trace of phosphorus. All protein tests were negative. It was insoluble in water and titration in alcohol showed it was not a free acid. With alcoholic potassium hydroxide a saponification equivalent of 380 was obtained, indicating an ester. The fatty acid isolated on saponification melted at about 40° and gave in alcohol a neutralization equivalent of 271.

The acetone extract (Sample 4) was dark brown and sticky. It contained nitrogen and phosphorus, but all protein tests were negative.

The absolute alcohol extract (Sample 5) was a brown, brittle material containing nitrogen and phosphorus, but all protein tests were negative.

Following the benzene extraction, the pneumococcus residue (Sample 7) was extracted for 3 successive days with three lots of distilled water, a few cc. of chloroform being added to preserve sterility. At the end of each 24 hours the solid matter was removed by centrifugalization and a fresh 250 cc. of distilled water were added. After the third extraction the combined water extract was run through the supercentrifuge to remove suspended matter. The milky, yellow extract gave strong specific precipitation with Type III immune serum and positive biuret, Millon's, xanthoproteic, *p*-dimethylaminobenzaldehyde, Ehrlich's diazo, and Molisch reactions. It contained practically all the soluble specific substance. (See Table II.) This extract (Sample 8) was divided into two equal parts of 375 cc. each and examined by the following methods, in order to determine in a rough way the amount of specific substance present in the organism.

Method 1.—The extract was treated with dilute acetic acid until on further addition of acid no more precipitation resulted. This precipitate (Sample 10) was removed by the centrifuge. It was washed virtually free from the specific substance with distilled water containing a little acetic acid. These washings were concentrated and added to the solution with 20 gm. of sodium acetate. Since a precipitate of specific substance could not be obtained directly with 1:1 hydrochloric acid, the solution at a volume of 425 cc. was precipitated with 1500 cc. of alcohol. The clear alcohol layer, showing no specific substance, was discarded. The precipitate was taken up in 75 cc. of hot water, acidified with a few drops of hydrochloric acid, and centrifugalized until clear. The solution was then treated with 40 cc. of 1:1 hydrochloric acid and, on standing in the ice box, the characteristic precipitate of the specific substance separated. It was removed by the centrifuge, redissolved in dilute sodium hydroxide, filtered through asbestos, after being made slightly acid with hydrochloric acid. On treatment with 50 cc. of 1:1 hydrochloric acid, after standing in the cold, the specific substance reprecipitated and was transferred to a hardened filter, washed with 50 per cent and 95 per cent alcohol free from chlorides, finally with acetone, and dried. The yield was 0.121 gm.

Method 2.—The remaining half of the water extract was evaporated to 250 cc. volume and precipitated with 800 cc. of alcohol. The precipitate was taken up in 300 cc. of hot water, 10 gm. of sodium acetate were added, and it was reprecipitated with 800 cc. of alcohol. The clear alcohol layers in both cases were discarded as the precipitin tests were negative. The precipitate was taken up in 150 cc. of hot water, centrifugalized, and precipitated with dilute acetic acid. This precipitate (Sample 12) was removed by the centrifuge and the clear solution acidified with 75 cc. of 1:1 hydrochloric acid when the specific substance precipitated (Sample 13). It was purified as in the former method except that it was filtered with considerable loss through a Mandler filter. The yield was 0.04 gm. As both lots of soluble specific substance (Samples 11 and 13) were equally pure, free from nitrogen, phosphorus, and ash, all protein tests being negative, the amount of this material in the pneumococci was calculated from the quantity isolated by Method 1. It amounted to 0.63 per cent

of the dry organisms. It agreed in properties exactly with that isolated from the medium.

The materials precipitated by dilute acetic acid from the water extract (Samples 10 and 12) were dried at 55°. The above color tests run on the water extract were positive.

The residue after water extraction (Sample 9) was further extracted with 600 cc. of 0.1 N sodium carbonate solution for 24 hours and the solid material (Sample 14) removed by the centrifuge. This material (Sample 14) was similarly extracted with 600 cc. of 0.1 N sodium hydroxide leaving a residue (Sample 17).

The sodium carbonate extract was acidified with acetic acid and a white, curdy precipitate (Sample 15) separated which was removed by the centrifuge, washed with very dilute acid, and dried at 55°. Virtually all the material extracted by the sodium carbonate was precipitated by the acetic acid. It amounted to 4.23 per cent of the initial material, was high in phosphorus, and gave the usual color tests.

The sodium hydroxide extract was similarly treated, acetic acid precipitating virtually all the extracted material (Sample 16). It was low in phosphorus, gave the usual color tests, and amounted to 21.1 per cent of the initial material.

The residue after the sodium hydroxide extract (Sample 17) was dark brown in color, high in ash content, due probably to adhering salts, and gave the usual color tests. Extraction was similarly continued with 200 cc. of 0.1 N acetic acid and then with 0.1 N hydrochloric acid for a 24 hour period, leaving the final residue (Sample 20) which was dark brown in color giving the usual color reactions, but negative Molisch reaction.

The acetic acid extract on evaporation left only sodium acetate with a trace of protein.

The hydrochloric acid extract on evaporation left a brown residue giving a pink color in the biuret reaction, an indication of proteoses, peptone, or protamines. The usual color reactions were positive, but the Molisch test was negative.

DISCUSSION.

These extractions resulted in the isolation of fractions of different chemical composition (see Table I). The absolute alcohol extract (Sample 5) contained 6.5 per cent nitrogen and 1 per cent

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phosphorus and was entirely free from protein. The water extraction removed material of high phosphorus content, probably of the nature of nucleoprotein, as well as the soluble specific sub-

TABLE II.
*Precipitin Test.**

Sam- ple No.	Material tested.	Antipneumococcus serum.			Nor- mal horse serum.
		Type I.	Type II.	Type III.	
1	Dried pneumococci (initial material).	—	—	++	—
2	Ether-soluble extract.	—	—	—	—
3	Residue after ether extraction.	—	—	++	—
4	Acetone-soluble extract.	—	—	—	—
5	Absolute alcohol “	—	—	—	—
7	Residue after extraction with ether, alcohol, acetone, and benzene.	—	—	+++	—
8	Water extract.	—	—	+++	—
9	Residue after water extraction.	—	—	+	—
10	Material precipitated by dilute acetic acid from water extract.	—	—	—	—
11	Soluble specific substance precipitated from water extract.	—	—	++++	—
13					
15	Material precipitated by dilute acetic acid from 0.1 N sodium carbonate extract.	—	—	—	—
16	Material precipitated by dilute acetic acid from 0.1 N sodium hydroxide extract.	—	—	—	—
17	Residue after 0.1 N sodium hydroxide extraction.	—	—	—	—
18	0.1 N acetic acid extract.	—	—	—	—
19	0.1 “ hydrochloric acid extract.	—	—	—	—
20	Final residue.	—	—	—	—
21	Physiological salt solution.	—	—	—	—

* The precipitin test was carried out by treating 0.3 cc. of the undiluted serum with 0.1 cc. of a saturated solution of the substances tested in physiological salt solution. Dilutions of these saturated solutions up to 1:1,000,000 were also tested with the same results. In one case the soluble specific substances Nos. 11 and 13 showed precipitation in dilutions as high as 1:1,000,000. The results of the test were read after the tubes had been heated at 37° for 3 hours and had stood in the ice box for 12 hours. Minus sign indicates no precipitation, + indicates very slight precipitation, ++ and +++ greater precipitation, and ++++ indicates precipitation given by pure soluble specific carbohydrate.

stance. Sodium carbonate also removed material of high phosphorus content, while sodium hydroxide of similar normality removed about 5 times as much material as the sodium carbonate, but it contained only a small amount of phosphorus. It is hoped that some of these fractions of such different chemical constitution will give biologic reactions of decided difference.

In Table II the results of the precipitin test with the various extracts are given. It shows clearly that the type specific precipitation was due to the soluble specific carbohydrate, as all the other fractions gave negative reactions except a faint reaction in the fractions, Samples 9, 10, and 12, which was undoubtedly due to a small amount of the specific substance adhering to the material. The residues, Samples 3 and 7, contained the specific substance, as it was not removed until the water extraction.

SUMMARY.

Virulent Type III pneumococci, after removal from the medium by a supercentrifuge and after being dried at 55°, have been analyzed for the elements nitrogen, phosphorus, sulfur, chlorine, ash, and volatile matter.

The soluble specific substance has been isolated from an 18 hour culture medium after removal of practically all of the pneumococci with a supercentrifuge, and a control run on the uninoculated medium indicated the specific substance so isolated to be entirely related to the pneumococci.

Extraction experiments with the dried pneumococci have resulted in the separation of ten fractions of different chemical composition, one of which was the soluble specific carbohydrate.

The fractions isolated have been tested by the precipitin reaction, and specific precipitation resulted only in the case of the soluble specific carbohydrate.

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THE INORGANIC COMPOSITION OF THE BODY FLUIDS OF THE CHELONIA.*

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In a previous paper analysis of the body fluids of some elasmobranch fishes were reported, which disclosed the fact that the pericardial and perivisceral fluids in these animals are highly specialized acid secretions (Smith (1)). These findings have led to the examination of some of the higher vertebrates to see if secretory activity on the part of the pericardial and perivisceral membranes is demonstrable in other animals.

Of the higher vertebrates, the turtles are unique, perhaps, in the relatively large quantities of pericardial and perivisceral fluids normally present. A 10 inch turtle may contain as much as 3 cc. of pericardial fluid and 6 cc. of perivisceral fluid, and in larger specimens the volume increases proportionally. The quantities of these fluids present in different individuals of the same species varies through wide ranges, however. In exceptional cases one or the other of these fluids may be entirely absent, while the other is present in considerable quantities.

Analyses of the CO₂ content of the serum of turtles have been made by Jolyet (2), Spallitta (3), Collip (4), and Southworth and Redfield (5). Collip and Southworth and Redfield point out that the CO₂ content is high in comparison with other vertebrates, their data ranging from 32 to 42 per mm per liter.

In spite of the high CO₂ content of the serum, the CO₂ content of the pericardial and perivisceral fluids rises to still greater values;

* Grateful acknowledgment is made to the Elizabeth Thompson Science Fund for assistance in part of the investigations reported here.

it is not uncommon to find in a single specimen, serum with 40 mm, perivisceral fluid with 80 mm, and pericardial fluid with 120 mm of CO_2 per liter. Since the total base remains approximately constant, the chloride diminishes as the CO_2 increases, and the pericardial fluid may, therefore, be a practically pure bicarbonate solution. Inasmuch as the CO_2 tension is probably the same in all the body fluids, the hydrogen ion concentration must decrease as the bicarbonate concentration increases, and these fluids become correspondingly more alkaline than the plasma. Such differences in composition, we suggest, are incompatible with the usual connotation of the term lymph, *i.e.* a protein-free ultrafiltrate from the plasma differing but little in its inorganic composition from the latter, and require a revaluation of the significance of these body fluids in the animals in which they occur.

The CO_2 content, hydrogen ion concentration, and chloride were determined directly on the fluids. SO_4 and PO_4 were determined on trichloroacetic acid filtrates prepared by adding 1 volume of 20 per cent trichloroacetic acid to 1 volume of serum, etc., plus 3 volumes of water. Na, K, Ca, and Mg were determined in redissolved aliquots of these filtrates after being ashed in quartz crucibles with H_2SO_4 , HNO_3 , and superoxol. The analytical procedures used have been listed in a previous paper (Smith (1)).

The operative procedures by which the fluids were removed are simple and require little comment. It is the usual custom to remove the perivisceral fluid first, from a dorsal entry to the peritoneal cavity. This approach affords the minimum amount of hemorrhage. A half-inch hole is trephined in the center of the posterior quadrant of the carapace and enlarged with bone-cutters until the posterior wall of the peritoneal cavity is freely uncovered. Any fluid found in the retroperitoneal connective tissue spaces is collected by aspiration into a syringe. The peritoneal cavity is then opened and the perivisceral fluid removed. Practically all the latter can be obtained from one side of the body if the animal is tilted slightly to one side.

The pericardial fluid is next removed from around the heart by trephining and cutting away part of the plastron. Some fluid may lie outside the pericardial cavity in the connective tissues under the plastron, in which case it is collected separately. The animal is then bled by aspiration from the venous sinuses and the blood

TABLE I.
Inorganic Composition of Body Fluids of Turtles.

Turtle No.	Sample.	mm per liter										pH
		Na	K	Ca	Mg	Cl	SO ₄	PO ₄	CO ₂	$\frac{+}{-}$ Na	$\frac{+}{-}$ Na	
1	<i>Chrysemys marginata belli</i> .											
	Composite, serum.	120	3 0	6 1	7 9	81 2		0 9	45 0	151	128	7.8
	“ perivisceral fluid.	134	3 3	3 4	1 4	64 5		0 7	74 5	147	140	8 1
	Composite, pericardial fluid	140	1 1	1 2	0 5	38 1		0 7	108 2	145	147	8 5
2	Composite, bile.	205	5 5	20 2	8 1	25 9			6 0	267	32 5	8
	<i>Chrysemys marginata belli</i> .											
	Composite, serum	149	3 6	4 8	1 6	89 20	8 1 0		47 7	165	140	7 80
	“ perivisceral fluid.	152	2 7	2 9	1 6	32 80	7 0 9		128 5	164	164	8 42
3	Composite, pericardial fluid.	152	2 3	0 8	0 9	25 0			130 5	158	156	8 50
	Composite, bile.	150	5 0	13 3	5 0	24 0			6 1	192	30 5	94
	<i>Graptemys geographica</i>											
	Composite, serum.	124	2 4	3 4	0 5	86 50	4 1 2		36 9	134	126	
4	“ perivisceral fluid.	122	2 4	3 3	0 6	68 00	8 0 9		64 6	132	136	
	<i>Emys blandingii</i> .											
	Composite, serum.	130	3 9	3 3	2 1	91 01	3 1 3		36 0	145	132	
	“ perivisceral fluid.	120	2 5	3 0	1 3	77 51	2 0 8		56 7	131	138	
5	Composite, pericardial fluid	132	2 6	1 3	0 5	62 2			75 5	138	138	
	<i>Pseudemys elegans</i> , ♀.											
	Serum.	126	3 3	4 2	4 4	80 80	1 1 4		42 0	147	125	
	Perivisceral fluid	133	2 6	3 2	3 3	41 70	1 0 8		87 0	149	130	
6	Pericardial “	138	2 1	1 5	0 8	15 0			120 0	145	135	
	Infracardial lymph	123	2 0	2 4	0 8	75 6			44.0	131	120	
	<i>Chelydra serpentina</i> , ♀.											
	Plasma.	141	3 5	6 5	4 5	54 50	3 1 0		44 5	167	101	7 75
	Perivisceral fluid.	151	4 1	5 2	4 2	56 60	3 1 1		59 5	174	118	7 85
	Pericardial “	141	3 4	4 5	2 0	50 50	4 0 9		67 0	157	120	7.92

TABLE I—Concluded.

Turtle No	Sample.	mm per liter.										pH
		Na	K	Ca	Mg	Cl	SO ₄	PO ₄	CO ₂	$\frac{H^+}{N}$	$\frac{A^-}{N}$	
7	<i>Chelydra serpentina</i> , ♂.											
	Plasma.	140	3.1	4.5	2.0	76.2	0.3	1.9	52.5	156	132	7.80
	Perivisceral fluid.	139	2.8	2.4	0.9	57.5	0.4	1.4	89.7	148	145	
	Pericardial "	140	3.0	2.5	0.3	15.5	0.3	1.5	130.9	149	149	
	Bile.	106	2.3	6.5	3.0	63	6	0.2	10.0	127	74	
8	<i>Caretta kemp</i> i, ♂.											
	Plasma.	163	6.6*	5.2	1.4	108.2	0.3	3.5	23.2	183	137	7.46
	Perivisceral fluid.	151	4.3	4.3	0.3	124.0	0.1	2.4	24.4	165	152	
	Pericardial "	151	4.6	4.1	0.3	129.0		2.0	29.0	164	161	
	Bile.								65	6		
9	<i>Caretta caretta</i> , ♀.											
	Plasma.	151	6.7	3.1	2.9	109.8		3.0	36.2	170	151	
	Perivisceral fluid.	129	4.3	3.1	5.0	114.2		2.4	34.8	150	152	
	Pericardial "	128	6.1	0.6	3.5	105.5		1.9	46.8	142	155	
	Bile.	113	8.9	12.8	10.4	26.4			7.5	168	34	

* Slight hemolysis.

defibrinated and centrifuged under oil. The bile can be aspirated from the gallbladder after complete removal of the plastron.

Analyses of the cerebrospinal fluid are not reported because we have not succeeded in removing the latter without considerable contamination with blood.

Inorganic analyses of several composite samples and, in a few instances, of body fluids removed from single specimens are given in Table I. Further data on the CO₂ content of body fluids from other individuals are given in Table II.

The fresh water turtles were kept in or near shallow fresh water and had been without food for periods varying from several weeks to several months. The marine turtles were obtained through the courtesy of the New York Aquarium. These had been eating up to a few days prior to examination.

Serum.—The serum presents little that is noteworthy, beyond the high bicarbonate content already noted by Collip (4) and Southworth and Redfield (5). There is generally a large excess of base over inorganic anions which in some instances (Turtle 6) may reach 33 per cent of the total base. The Mg content is in general

much higher than in mammals, though SO_4 and PO_4 do not exceed the usual mammalian level.

Perivisceral Fluid.—The most notable features of the perivisceral fluid are its high CO_2 content and equivalently low Cl. There is wide variation in this respect, some animals showing only a slightly

TABLE II.
Distribution of CO_2 in Body Fluids of Turtles.

Turtle No.	Sample.	mm CO_2 per liter.					
		Serum.	Lymph.	Perivisceral fluid.	Pericardial fluid.	Bile.	Urine.
10	<i>Graptemys geographica</i> , ♀.	37.7		58.3	72.2		13.5
11	" " ♀.	38.2		69.9	103.0		5.7
12	" " ♀.	33.1		69.6	68.8		5.9
13	" " ♀.	48.1		75.6	94.8		6.1
14	" " ♀.	38.3		52.8			7.2
15	<i>Chrysemys marginata belli</i> , ♀.	43.9			92.4		
16	" " ♀.	48.3		127.3	138.5		
17	" " ♀.	48.9		109.0	148.5		
18	" " ♀.	47.6		125.1	134.0	7.0	
19	" " ♀.	49.3		143.3	134.4	0.6	4.2
20	<i>Emys blandingii</i> , ♂.	36.6		53.2	82.3		
21	" " ♂.	42.5		43.7	61.3		
22	" " ♂.	37.0		42.7	41.4		
23	" " ♀.	43.1	40.0	91.0	116.0		
24	<i>Pseudemys elegans</i> , ♂.	43.3		95.0	118.0	0.4	1.8
25	" " ♀.	40.5	40.1	86.5	120.0	0.4	1.3
26	" " ♀.	44.5	42.1	79.7	121.0	0.35	1.7
27	<i>Kinosternon subrubrum</i> , ♀.	29.8		38.0	50.0		
28	<i>Chelydra serpentina</i> , ♀.	45.0	44.6		98.0		
29	<i>Caretta kemp</i> .	28.0		30.4	32.8		
30	" "	32.0		32.2	37.2		
31	" "	30.0		33.0			
32	" "	30.6		32.0	35.0		

higher CO_2 content than the serum, while others of the same species show 3 times as much. The total base in serum and perivisceral fluid is about the same, and the increase in CO_2 is offset by an equivalent decrease in Cl.

Ca and Mg, when they differ from the serum level, run below

the latter. K varies both above and below the serum level, though the relative deviation is not so marked as in the case of Ca and Mg. As a rule there is little if any excess base over inorganic anions in this and in the pericardial fluid as compared with serum.

It is noteworthy that in Turtle 6, the excess base in all the body fluids reaches a maximum, while the HCO_3 is markedly reduced, suggesting displacement of the latter by an organic acid.

The high HCO_3 content of the perivisceral fluid is all the more striking in view of the protruberance into the peritoneal cavity of the large and relatively thin-walled urinary bladder and the gallbladder, both of which may contain fluids having a much lower HCO_3 content than the blood. Since all the viscera are covered by the reflected peritoneum, they lie outside this cavity in the physiological sense.

Pericardial Fluid.—In this, as in the perivisceral fluid, the most striking feature is the extraordinary CO_2 content, which may exceed that of the serum threefold; five-sixths of the total salt present in the pericardial fluid may be bicarbonate. In general the CO_2 in the pericardial fluid is higher than in the perivisceral fluid; the only recorded exceptions to this sequence are Turtles 12, 19, and 22.

Other tendencies evident in the perivisceral fluid are exaggerated in the pericardial fluid. The Ca and Mg show further reductions, reaching such extreme serum ratios as 1:8 and 1:15, respectively. The K in the pericardial fluid, as in the perivisceral fluid, shows a tendency to deviate less from the serum level than do Ca and Mg. This distribution of K in the body fluids of turtles is in marked contrast to the situation in the elasmobranchs, in which the K of the pericardial fluid may exceed the serum level by several fold.

Lymph.—True lymphatic channels occur in these animals, but we have never succeeded in removing fluid from them. But a transudate is sometimes observed in the retroperitoneal or infracardial connective tissue spaces, which, it is to be noted, lies outside the true body cavities. It is characterized by possessing practically the same CO_2 content as the serum, in marked contrast to the fluid within the peritoneal and the pericardial cavities. Sufficient quantities were obtained to permit complete analysis in only one instance. Although in this instance the Ca and Mg

deviate markedly from the serum, the relatively equal distribution of CO_2 between the serum and this fluid in all cases in which it was observed leads us to designate it as lymph, implying that it is a passive transudate from the plasma, a non-specialized ultrafiltrate. The occurrence of a fluid of this nature in these animals emphasizes all the more the specialized, secretory nature of the pericardial and perivisceral fluids.

The present evidence contributes little toward explaining what the function of these fluids might be. An obvious suggestion is that the high HCO_3 furnishes additional alkali reserve to offset the accumulation of acids other than carbonic acid during either a period of terrestrial hibernation or prolonged submergence in water. The fact that the HCO_3 is lower and more uniformly distributed in the marine turtles (*Caretta kempfi* and *Caretta caretta*) which do not hibernate suggests that specialization in these fluids is related to terrestrial or semiterrestrial life. It may be noted that some, though not all, of the semiterrestrial turtles studied can carry on subaquatic respiration by drawing water into the mouth, and this accessory respiration enables them to remain submerged for long periods. The process of respiration in these forms has not been extensively studied, however, and to link the high bicarbonate of the body fluids with prolonged submergence, or with hibernation, is at best a speculation.

Comparison of Turtles 3, 6, and 8 with the others indicates that the Ca content of these fluids, and to a lesser extent the Mg content, tends to vary inversely with the HCO_3 content.

It would be expected that an increase in HCO_3 would tend to reduce the Ca (and Mg) by virtue of the insolubility of the carbonates of these substances, or at least to establish an upper limit, at a fixed CO_2 tension, to the quantities of them present in solution, especially in view of the fact that the pericardial and peritoneal fluids contain considerably less protein than the serum and therefore offer less opportunity for the organic binding of Ca. But variations in the Ca content cannot safely be attributed solely to the stoichiometric relations of the constituents of these fluids, since the Ca content is similarly reduced, and to an even greater extent relative to the serum, in the acid perivisceral and pericardial fluids of the elasmobranchs. In the latter instance, the reduction in Ca content cannot be explained in any other terms

than as an expression of the secretory nature of the peritoneal and pericardial membranes; and in view of the demonstrable secretory nature of these membranes in the turtles, the divergent Ca content of the pericardial and perivisceral fluids in these animals must be viewed with suspicion.

A significant fact relative to the composition of these fluids rich in HCO_3 is the corresponding diminution in Cl. This fact arises from the circumstances that (1) the perivisceral and pericardial fluids are approximately isotonic with the plasma and (2) the osmotic pressure in them is furnished almost entirely by monovalent, inorganic constituents; under which circumstances the total base is approximately the same in all the body fluids. Hence any increase in HCO_3 must be offset by an equivalent decrease in the concentrations of other anions, and Cl being present in predominant amounts, this substance shows the decrease most markedly.

In this view the inequality in the distribution of Cl in these body fluids is not of primary significance, but follows from the equality of total base and the fact of increased HCO_3 concentration. The essential problem here, then, involves the unequal distribution of HCO_3 . Concerning this distribution of HCO_3 there are several considerations which may be briefly noted and which apply, we believe, to the acid and alkaline secretions of vertebrates in general.

It is a widely accepted belief that all living tissues are permeated with relative ease by carbonic acid. (Reasons have been given for supposing that it is the anhydride, CO_2 , rather than the acid H_2CO_3 , which migrates so easily through protoplasmic barriers which are capable of restricting the migration of other molecular species (Smith and Clowes (6)). Excluding those foci where CO_2 is being formed *de novo*, this rapid penetration results in a practically uniform distribution of this substance throughout the body. No factors are known which can lead to significant inequality in the equilibrium distribution of a molecular species between two similar aqueous solutions separated by a membrane permeable to that species. (We use the terms "similar aqueous solutions" to exclude such theoretically possible, but practically inconsiderable differences in solubility or partial pressure as might result from differences in salt content, temperature, etc.) From

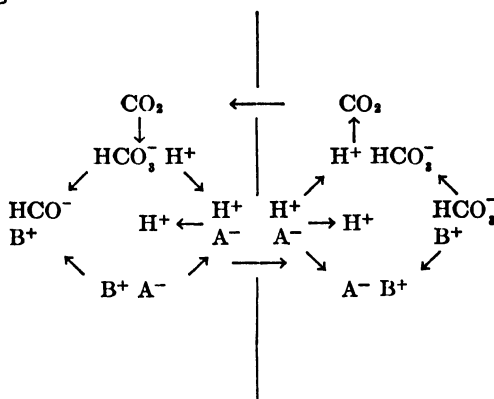
the similarity in composition of the various body fluids it may be supposed that the CO_2 will be equally hydrated ($\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$) in them, and, consequently, the acid moiety (H_2CO_3) will also be distributed equally. It is also to be expected from this similarity in composition that the dissociation constant of H_2CO_3 and the activity coefficient of HCO_3 will be approximately the same in the several body fluids.

Since the HCO_3 and Cl content of these and other body fluids are subject to variation, the parent membranes from which these fluids arise must be capable of transporting from the blood one or both of these ions. But in the fluids under consideration, and in the gastrointestinal secretions, urine, etc., of vertebrates generally, the distribution of both HCO_3 and Cl between the parent plasma and the elaborated fluid is of such a nature as to exclude the possibility of free, unimpeded exchange of anions across these membranes.

It follows that the observed differences in composition of these fluids with respect to HCO_3 , Cl , and H^+ , can only arise by the mediation of the membranes elaborating them in specifically conditioning the distribution of one or more of these ions. So far as HCO_3 and H^+ are concerned, it is impossible and inconsequential to distinguish which is the effective ion in relation to the membrane because, by the mass law, they are dependent variables referable to the acid moiety.

But it remains to be determined whether both HCO_3 and Cl , or only one of these ions, is conditionally transported by the membrane. Evidence has been given (Smith (7)) that in some other tissues the penetration of anions depends upon the prior penetration of the acid (CO_2 , etc.); and the suggestion has been made that in consequence of changes in the H^+ concentration gradient across the membrane there occurs an active transportation of anions in general (and of Cl in particular since it is most abundant) across the membrane, leaving B^+ free to combine with the penetrating acid. Thus, in contradistinction to the view that HCO_3 and Cl are freely exchanged by diffusion, emphasis is placed on the sequence: (1) migration of the CO_2 , (2) active transportation of anions in general and Cl in particular, and (3) combination of CO_2 with residual base to form bicarbonate. Thus the HCO_3 concentration is established by the migration of CO_2 in one direction, and the migration

of H^+ and Cl^- in the opposite direction, the latter process being initiated and regulated by differences in H^+ concentration on the two sides of the membrane. This sequence is illustrated in the following scheme.



Whether this sequence applies to the transportation of anions by other tissues remains to be determined. But the fact that HCO_3^- and Cl^- are in all instances of secretion distinctively distributed indicates that these ions are handled in some such separate and distinctive manner.

This view is not at variance with the fundamental principles relating to osmotic pressure and the Donnan effect which have been applied so successfully to the distribution of HCO_3^- and Cl^- between mammalian red blood cells and plasma by Van Slyke and his coworkers, but adds to these principles the active participation by the membrane in the transportation and the determination of the equilibrium distribution of anions. This addition appears to us to be necessary before any approach can be made to the phenomena of secretion of acid and alkaline fluids. The concept is consonant with the known facts of secretion and anion transportation, and offers a tentative basis for interpreting these facts, as well as the bicarbonate "thresholds" of the kidney, etc., by recognizing that it is the particular characteristics, or orientation of the membrane, as well as the composition of the fluids on either side, that determine whether the fluid on one side of the membrane shall be more acid, more alkaline, or identical with the fluid on the other side after the transporting membrane has come to rest in the equilibrium state.

SUMMARY.

The perivisceral and pericardial fluids of the Chelonia are alkaline secretions in which the bicarbonate content may be 200 to 300 per cent higher than in the plasma. The total base in these fluids and in the serum is approximately the same; consequently the chloride is diminished by approximately equivalent amounts as the bicarbonate is increased.

Potassium, calcium, and magnesium are occasionally present in the perivisceral and pericardial fluids in considerably smaller amounts than in serum.

In some specimens a transudate is present in the retroperitoneal or infracardial spaces which has approximately the same bicarbonate content as the serum, and which may be appropriately designated as lymph, in the sense of a simple ultrafiltrate from the plasma.

Emphasis is placed upon the secretory properties of the pericardial and peritoneal membranes in these animals.

Some general considerations pertinent to the secretion of acid and alkaline solutions are discussed.

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THE METABOLISM OF SULFUR.

XVI. DIETARY FACTORS IN RELATION TO THE CHEMICAL COMPOSITION OF THE HAIR OF THE YOUNG WHITE RAT.

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Notable in the chemical composition of the proteins of the hair (and other epidermal structures) are the high contents of cystine and sulfur (1). In a previous study (2), we have been able to demonstrate that, in the young white rat, *the amount of hair produced was related to the protein (and cystine) content of the diet*, but that the demands for protein (and cystine) for the growth of the hair appeared to be secondary in importance to the demands for growth of the body with its more essential tissues.

In continuation of these studies, the present investigation is concerned with the *composition of the hair as related to dietary factors*. Are the amounts of cystine and sulfur present in hair influenced by factors of age or diet? Is the chemical composition of the protein of a tissue constant, or does the composition of a protein elaborated under conditions of a shortage of a specific amino acid reflect the lack of that particular amino acid in the diet? Since the hair is notably high in its content of cystine and since it has been demonstrated that cystine is used preferentially for general somatic development rather than for the growth of the hair under conditions of inadequate protein (and cystine) content of the diet (2), it has seemed that an attempt to solve this general question of the constancy of the composition of a tissue protein might well be made by a study of the composition of the hair produced by the young white rat on diets of varying protein and cystine content.

The general plan of the experiments was similar to that previously reported (2). Young white rats at the age of 30 days were selected from our colony in such a way that litter mates were distributed between the diets chosen, in order to eliminate so far as possible variations between litters. The animals were weighed weekly during an experimental period of 10 weeks. At the end of the 10 week period, the hair was collected by clipping. The samples were extracted with absolute alcohol and ether for periods of 24 hours each, dried at 60° *in vacuo*, and stored until analyzed in a desiccator over calcium chloride. Total sulfur was determined gravimetrically after oxidation of the sample in the Parr bomb; cystine, by the colorimetric method of Folin and Looney; and total nitrogen, by the Scales and Harrison modification of the Kjeldahl method (3). Duplicate analyses were carried out, when the amount of material available permitted; with the smaller animals, however, this was often impossible.

For the basis of the diets in which the amount of cystine present was the limiting factor, the milk powder-starch diet of Sherman and Woods (4) was used, modified as in previous studies (2). Groups 9 and 10 received diets in which 0.47 and 16 per cent respectively of casein replaced equivalent amounts of dextrin. In order to demonstrate more clearly that we were concerned with the quantity of cystine and not with the quantity of protein as such in these diets, the animals of Group 12 received a diet in which 6 per cent casein and 0.25 per cent of cystine replaced an equivalent amount of dextrin. The diets of Groups 10 and 12 should have contained approximately equal amounts of cystine,¹ although differing considerably in their content of total protein (casein).

As further controls, collections of hair were also made from rats fed the stock diet previously described (2) for 10 weeks (Group 11), from rats at the age of 30 days (Group 15), from animals fed

¹ Sherman and Woods (4) in their biological test of the cystine content of casein found that 1 gm. of casein was equivalent in its effect on growth to 0.025 gm. of cystine. Hence the addition of 0.25 per cent of cystine to 6 per cent casein (Group 12) should give a cystine content equivalent in *biological value* to 16 per cent casein (Group 10). On the basis of the *chemical analyses* (5), however, in which casein was found to contain only 0.25 per cent of cystine, the diet of Group 12 would have included much more cystine than the diet of Group 10.

TABLE I.
Composition of Hair.

Group No.	Diet.	Final weight, average.	Composition of hair of individual animals, per cent.	Average for group.
9	Casein, 0.47 per cent. •	106.5 <i>gm.</i>	S. 3.80, 3.86, 3.98, 4.21, 4.03, 3.74, 3.74 Cystine. 10.96, 10.82, 11.79, 11.99, 11.47, 11.36, 11.50 N. 15.8, 16.0, 17.2, 17.0, 16.9	3.90 11.41
10	Casein, 16.0 per cent.	203.9	S. 4.27, 4.58, 4.31, 4.22, 4.43, 4.21, 4.68 Cystine. 12.36, 12.61, 12.89, 12.68, 12.99, 13.02, 12.79 N. 16.3, 16.1, 15.5, 16.2, 16.2, 16.9, 16.4	4.39 12.76
11	Stock diet.	199.5	S. 4.58, 4.28, 4.11, 3.89, 3.93, 4.64, 4.96 Cystine. 12.83, 12.68, 12.99, 12.27, 12.88, 12.52, 13.02 N. 15.5, 15.9, 15.2, 15.9, 15.7, 15.9, 16.1	4.34 12.74
12	Casein, 6.0 per cent. Cystine, 0.25 per cent.	181.7	S. 4.47, 4.59, 4.21, 4.30, 4.25, 4.52, 4.41, 4.34, 4.97, 4.73 Cystine. 12.48, 13.15, 12.78, 12.01, 12.24, 12.82, 12.87, 12.02, 11.82, 13.75	4.48 12.59
13	Low lysine.	124.9	S. 4.21, 3.98, 4.53, 4.50, 3.89, 4.96, 3.98, 4.15, 4.88 Cystine. 12.70, 12.46, 12.75, 12.88, 12.75, 12.40, 12.23, 12.29, 12.29, 12.77	4.34 12.59
14	Average weight, 120 gm.	118.9	S. 3.87, 3.96, 3.43, 3.22, 3.55, 3.37, 4.24 Cystine. 10.01, 10.80, 10.82, 11.01, 10.95, 11.67, 10.97, 10.90 N. 16.6, 17.0, 16.6, 16.8, 16.9, 16.6, 16.6, 16.4	3.66 10.98

TABLE I—*Concluded.*

Group No.	Diet.	Final weight, average.	Composition of hair, of individual animals, per cent.	Average for group.
		<i>gm.</i>		
15	Age, 30 days.	65 5	S. 3.72, 3.45, 3.28, 3.76, 3.93, 4.04, 4.02, 3.88 Cystine. 11.23, 11.02, 10.19, 10.19, 10.24, 10.37, 10.00, 11.32, 10.16 N. 17.0, 16.8, 16.3, 17.1, 16.7, 17.0, 17.2, 16.7	3 76 10 52
16	Adults.	281 8	S. 4.09, 4.60, 4.67, 4.32, 4.78, 4.46, 4.63, 4.59, 4.57, 4.36, 4.82 Cystine. 11.73, 12.21, 13.09, 13.68, 13.58, 14.63, 12.16, 12.62, 12.25, 12.05, 12.86 N. 15.6, 16.1, 16.0, 16.6, 16.5, 15.9, 15.8	4 53 12 80

the stock diet (for a period of 2 to 4 weeks) until they reached an approximate body weight of 120 gm. (Group 14), and from a group of stock adult rats (Group 16). In order to further control our experiments with diets varying in their content of cystine, hair was collected for analysis from a group of animals (Group 13) which were fed a diet which retarded growth as did the cystine-deficient diet, but which was inadequate in respect to a different amino acid, lysine. The lysine-deficient diet (6), upon which these animals were maintained for a period of 10 weeks was made up of rolled oats (60.0 per cent), dextrin (30.3 per cent), salt mixture (4.7 per cent), and butter fat (5.0 per cent). Animals on this diet showed a retarded growth similar to that of the animals of Group 9, which received a diet in which cystine was the factor limiting the rate of growth.

As shown in Table I, the analytical data fall into two general groups. All of the animals fed a diet adequate in its cystine content over an experimental period of 10 weeks had produced hair which contained nearly the same percentage of cystine (Groups 10 to 13), a figure which was comparable also to the cystine content of the hair of the adult animals (Group 16) which were from 12 to 18 months old. On the other hand, the animals of Group 9,

whose growth was limited by the low cystine content of the diet and which in a period of 10 weeks on the experimental diet were unable to make as great a gain in weight as animals on the stock diet in 2 to 3 weeks (Group 14), have produced hair which was distinctly lower in its cystine content than that produced in the same period of time by the animals fed diets adequate in their contents of cystine as already discussed.

That the lower cystine content of the hair of the animals receiving amounts of cystine insufficient to permit a normal rate of growth was not the result of retardation of growth *per se*, however, is demonstrated by the cystine content of the hair of the rats which also received a diet inadequate for a normal rate of growth, but in which the amount of lysine rather than of cystine in the diet was the limiting factor (Group 13). Although the animals of this group were able to make increments in weight only slightly greater than the animals of Group 9, the hair produced during this period was similar in its cystine content to that of those animals which received amounts of dietary protein and cystine such that marked growth resulted.

The cystine content of the hair of the rats receiving the diet inadequate in its cystine content was similar to and only slightly higher than the cystine content of the hair of the young rats (Group 15), animals whose hair was obtained for analysis before the development of the adult type of hair (7). The animals of Group 14 from which hair was collected during the period of transition from the "puppy" coat to the adult type, and which were fed the stock diet for 2 to 3 weeks only also showed a lower content of cystine in the hair.

The total sulfur content of the hair showed variations similar to and parallel to the cystine content, since it was possible in this case also to distinguish between two types of hair, the low sulfur type (Groups 9, 14, 15) and a higher sulfur type (Groups 10 to 13, 16). That these differences in sulfur and cystine content were not due to differences in content of moisture, ash, or other constituents, was shown by the analyses for total nitrogen which varied little throughout the series. As a matter of fact, the nitrogen content of the hair of Group 15, which showed the lowest average cystine content of all the groups, would appear to be slightly higher than that of any other group.

From these results, it would seem that the initial coat of hair ("puppy" coat) contains a lower content of cystine than the adult coat;² that during the early period of rapid somatic development, although a heavier coat of hair (adult hair) may be produced (2), the cystine content of the hair does not increase significantly; but that in the later stage of somatic development, in which growth proceeds less rapidly, if adequate amounts of cystine are available in the diet, the cystine content of the hair is increased. During the longer period of time, if the cystine content of the diet is not sufficient for either normal somatic development or normal growth of the hair, the percentage of cystine in the hair changes little from that of the "puppy" coat of hair. Retardation of growth, due to a shortage of dietary factors other than cystine, *e.g.* lysine, does not affect the cystine content of the hair, which resembles that of the adult type of hair.

In the series considered, the animals fed experimental diets were for the most part, *of the same age but of widely different body weights* at the time the hair was collected for analysis. It might be argued that age and body weight were more important factors in determining the cystine content of the hair than were dietary factors. In a second series, the animals were placed on a series of experimental diets, but the hair was collected from each animal when it had reached a body weight of approximately 120 gm. In other words, we were concerned with the analysis of hair produced by animals *of the same body weight but of different ages*. The stage of somatic development corresponding to a body weight of 120 gm. was chosen since, in our colony, this weight in control animals was reached at the age of 6 to 7 weeks, the time at which according to Greenman and Duhring (7) the transformation from "puppy" to adult hair has occurred.

The diets of the second series were similar to the preceding. A control group (Group 17) received the stock diet, a second group (Group 18) received the Sherman-Woods diet in which 16 per cent of casein had been substituted for an equivalent amount of carbo-

² Düring (8) observed that the total sulfur content of the hair of rabbits remained relatively constant until the 3rd month, and that the total sulfur increased slightly thereafter as the animals became older. The changes, 4.00 per cent to 4.65 per cent of total sulfur, are similar to those we have observed in the composition of the hair of rats.

hydrate, a third group (Group 19) received the same diet as Group 18 with the further addition of 0.75 per cent of cystine to insure an excess of cystine in the diet. The rate of growth of these three groups, as shown in Table II, was essentially the same, since all animals of these groups had attained a weight of about 120 gm. at the age of 6 to 7 weeks (*i.e.*, after 2 to 3 weeks on the experimental diets).

Two groups received diets which retarded growth. The animals of Group 20 received the basal Sherman-Woods diet as modified

TABLE II.
Cystine Content of Hair.

Group No.	Diet.	Age.	Final weight, average.	Cystine content of hair of individual animals, per cent.	Average for group.
		<i>wks.</i>	<i>gm.</i>		
17	Stock diet.	7	122.8	11.40, 11.44, 12.26, 12.52, 11.38, 12.41	11.90
18	Casein, 16.0 per cent.	6-7	125.0	13.51, 13.82, 15.44, 15.28, 14.29, 14.07, 15.03	14.49
19	Casein, 16.0 per cent; cystine, 0.75 per cent.	6-7	121.4	14.06, 15.14, 14.29, 14.80, 14.51, 15.16, 14.76	14.67
20	No added casein.	11-15	119.0	10.08, 10.05, 10.77, 9.85, 10.93, 10.72, 10.48	10.41
21	Low lysine.	12-14	123.8	12.51, 12.46, 12.75, 12.09, 12.50, 12.46, 12.20	12.42

(2) with no supplementary casein. For the low lysine diet of this series, the gliadin diet of Osborne and Mendel (9) was used. This diet contained gliadin, 18 per cent; salt mixture, 4.5 per cent; dextrin, 50.0 per cent; lard, 24.5 per cent; and cod liver oil, 3.0 per cent.³ On these two diets growth proceeded slowly and an average body weight of 120 gm. was reached at the age of 11 to 15 weeks (*i.e.*, after 7 to 11 weeks on the experimental diets).

The analytical data for this series demonstrate even more strik-

³ Vitamin B was supplied by Vegex in amounts of about 75 mg. daily per rat.

ingly the influence of the cystine content of the diet on the composition of the hair (Table II). The animals of Groups 18 and 19, which were supplied with amounts of cystine fully adequate for or in excess of the requirements for rapid growth, produced hair which contained a high percentage of cystine. There was no significant difference in the composition of the hair of those animals which received excess cystine in the diet (Group 19), thus demonstrating that amounts of cystine in excess of those necessary to meet the demands for growth and to insure the production of hair of normal composition did not result in the production of hair of proportionately greater cystine content. The animals of Group 20, which, in a period of 11 to 15 weeks, *i.e.* nearly twice the time required by the animals of Groups 18 and 19, had reached a body weight of about 120 gm. and which as shown previously had been able to add little hair to their coat in this period (2), had elaborated a coat of hair much lower in its content of cystine, similar in cystine content to the hair of young animals of the age of 30 days (Table I). The animals of Group 21 which also grew slowly due to a dietary deficiency of lysine and whose diet contained cystine in adequate but not excessive amounts, had produced a hair whose cystine content was significantly greater than that of the animals on a cystine-poor diet, a cystine content similar to but slightly higher than that of the stock diet group (Group 17) which made a normal rate of growth.

These results supplement the data of the first series of experiments (Table I). They indicate that in the period of rapid growth hair of a cystine content similar to that of adults may be produced if excess cystine is present in the diet, and that if an adequate but not excessive amount of cystine is furnished by the diet in this early period of growth, the hair may contain a higher percentage of cystine than that produced by young animals, but that under these circumstances, the cystine content of the hair is not as great as in the adult or in the younger animals which have received an excess of cystine in the diet.

It should be noted that Group 10 (Table I) and Group 18 (Table II), both of which received the same diet showed a considerable difference in the percentage of cystine present in the hair. We are unable to determine whether this is a variation due to individuals, season, or age. The experiments with the groups presented

in Table II were carried out subsequent to those in Table I. The rats of Group 18 were from 6 to 7 weeks of age while those animals of the earlier series were about 14 weeks of age. Experiments designed to study more in detail the effect of these and other factors on the composition of the hair have been planned.

SUMMARY.

1. Further evidence is presented which indicates that the cystine requirements for growth of the body take precedence over the cystine requirements for the production of hair in the white rat.

2. The cystine and sulfur contents of the hair of the white rat were found to vary with the cystine content of the diet and to some extent with the age of the animals.

3. The cystine and sulfur contents of the hair of young rats ("puppy" coat) were significantly lower than those of the hair of rats fed on diets high in their content of cystine.

4. Hair from animals which received a diet in which cystine was the factor limiting growth, resembled in its lower cystine and sulfur contents the first coat of hair produced in young rats.

5. Retardation of growth alone did not produce a hair low in its cystine content, since a diet deficient in some other factor than cystine, *e.g.* lysine, did not result in the production of a hair of abnormally low content of cystine or sulfur.

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ON THE QUESTION OF THE ORIGIN OF URINARY AMMONIA.

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A few years ago we (1) presented evidence in favor of the view that urinary ammonia is formed by the kidney and that acid is not neutralized by ammonia during transportation in the blood. In subsequent communications (2) we discussed later experimental findings of others bearing on the question of the origin of urinary ammonia. During the past 2 years further work has appeared which is more or less directly related to this question and we feel that a discussion of some of these recent findings is desirable. Embden (3) and Parnas and Mozołowski (4) have studied ammonia formation in muscle tissue in relation to contraction, and later Embden and his collaborators reported extensive studies along this line (5). Recently Bliss (6-8) has reported work related to the problem of the origin of urinary ammonia.

Bliss has frankly adopted our view that the traces of ammonia found in the blood are of no significance in connection with the neutralization of acids in the body. Obviously this view involves acceptance of the theory that the urinary ammonia has its origin in the kidney. Bliss has presented evidence from which he concludes that the amide nitrogen fraction in blood protein is subject to quantitative variation under certain definite conditions. As a result of this work and the further finding that the kidney possesses an enzyme capable of liberating ammonia from the amide nitrogen of the blood proteins, Bliss draws the inference that this amide nitrogen fraction is closely related to the process of neutralization of acids in the tissues, or at least to the neutralization of lactic acid in muscle, and to the ammonia which is found in the urine.

In connection with the presentation of his work Bliss has frequently made statements which we feel may tend to mislead the reader in regard to the real significance of what he reports. The following is quoted from one of Bliss' recent papers ((7) p. 130).

"The excessively low values for the ammonia of blood lead one to suspect that ammonia which is formed for acid neutralization in the tissues may be masked in its transit through the blood to the kidney for excretion. . . .

"The behavior of compounds of glucose and ammonia is suggestive. The writer has prepared crystalline compounds of glucose and ammonia . . . , and found that when this substance is subjected to the ordinary aeration method for determining the ammonia content of blood only 1 per cent, or less, of the nitrogen is obtained as ammonia. However, a few minutes contact with 0.1 N acid is sufficient to free the ammonia, and aerations of samples so treated yield 100 per cent of their nitrogen as ammonia by the direct aeration method as used for blood."

We disagree with the view here expressed that the behavior of glucose-ammonia condensation products is suggestive in connection with the problem of transportation of ammonia in blood. In our first paper on ammonia formation we showed, through the addition of large amounts of lactic acid to the blood, followed by the failure to find any increase in ammonia, that there was no compound present in blood which would yield ammonia for acid neutralization when treated with an acid which is a normal physiological compound. Later in the same paper Bliss refers repeatedly to an ammonia complex in the blood. We deem the use of this term "ammonia complex" unjustifiable and misleading. An amide firmly bound in protein and requiring 90 minutes heating with 4 N sulfuric acid for its hydrolysis to ammonia is certainly no more an ammonia complex than is, for example, urea.

In his papers which have appeared so far Bliss stresses the neutralization of lactic acid in the muscle by means of ammonia. He assumes that just as soon as the ammonia reaches the blood stream it is detectable only as firmly bound protein amide nitrogen. Bliss realizes that if this view is adopted, it is necessary to postulate the existence of ammonia in tissues, and he makes the following statement: "So far as the writer is aware, the presence of ammonia in tissues has never been seriously questioned" ((8) p. 139). Bliss then cites two references to work carried out from

25 to 35 years ago and includes no reference to the clear cut more recent experiments of Gad-Andresen who found that in a number of tissues, including muscle, the concentration of ammonia is exactly the same as it is in the blood. In view of these findings of Gad-Andresen, we had not given serious consideration to the possibility of acid neutralization by ammonia within the tissues.

The question of the neutralization of lactic acid in muscular tissue by means of ammonia has been studied by several investigators prior to Bliss' indirect work on this subject. In 1927, Embden (3) and Parnas and Mozolowski (4) both reported an increase in ammonia content of muscle tissue following work, and later Embden and his collaborators reported extensive studies along this line. Parnas and Mozolowski believed there was a relationship between the lactic acid and ammonia content of the muscle, indicating a neutralization of the one by the other. Embden and Wassermeyer on the contrary (9) failed to find any such relationship.

Bliss' study of this question is indirect and we should hesitate to conclude with Bliss that because an increase in amide nitrogen may be demonstrated in the blood circulating through muscle tissue following exercise, there is a neutralization of lactic acid within the muscle by means of ammonia. A direct study of the question by means of determinations of the ammonia content of muscle under varying conditions would appear to be the desirable mode of attack in a study of this problem.

The figures reported by Embden and his coworkers for the ammonia content of frog muscle are approximately twice as high as the values found by Gad-Andresen. This latter investigator cooled the muscle tissue to -20° immediately after its excision. Embden and his coworkers on the contrary appear to have made little effort to maintain the muscle tissue at low temperatures during its preparation for analysis. It would seem possible that the higher acid concentration of exercised muscle might lead to more rapid postmortem ammonia formation. Unfortunately the actual technique for the determination of ammonia employed by Gad-Andresen is not available. Embden and his coworkers used a complicated procedure involving distillation of the tissue with magnesium oxide at temperatures of $35-38^{\circ}$, the ammonia being received in 0.03 N sulfuric acid. A double titration with iodine

and thiosulfate was then employed to determine the amount of acid neutralized. In spite of this indirect and complicated method of analysis, Embden, Riebeling, and Selter (5) reported a most astonishing agreement in a series of figures for the ammonia content of the right and left leg muscles from the same frog. The maximal difference reported in their table is 0.05 mg. of ammonia per 100 gm. of muscle with an average variation of less than half this figure. Our experience with ammonia determinations leads us to state frankly that the apparent degree of accuracy of the method employed by Embden, Riebeling, and Selter is quite beyond our comprehension.

Obviously the question of ammonia formation in muscle during exercise is in no way settled at the present time. Bliss' finding of increased amide nitrogen in the blood following exercise is a type of indirect evidence which cannot be accepted as conclusive. Even assuming such ammonia formation in muscle during work, Bliss' position that the ammonia appears in the blood stream as firmly bound amide nitrogen is a corollary which we feel many could not accept. How the ammonium ion on one side of the cell membrane appears as firmly bound protein amide nitrogen on the other side, it is difficult to imagine. We would point out that Bliss' latest position in regard to the transportation of acids in the blood and the origin of urinary ammonia is practically in exact accord with our own views. Since the amide nitrogen is firmly bound in the protein molecule and the acids are transported in the non-protein portion of the blood, it is obvious that there is no direct neutralization of acid transported in the blood by means of ammonia. Furthermore, as a corollary of Bliss' present position, there is the direct inference that the urinary ammonia arises through action of the kidney on a non-ammonia precursor in the blood exactly as we suggested. It may be noted in this connection that we suggested an amide, namely urea, as the probable source of the urinary ammonia, though we offered no evidence that urea was the particular compound involved. Whether urea or some other non-ammonium compound is the source of urinary ammonia does not affect the fundamentals of the theory. We would point out, however, that the finding of an enzyme in the kidney capable of splitting ammonia from protein amide does not constitute evidence that urea is not the source of urinary ammonia.

Urea being a highly diffusible compound, this amide would presumably be more available as a source of ammonia during the rapid passage of blood through the kidney than would amide nitrogen which is firmly bound in the non-diffusible protein molecule in the blood. The fact that the kidney can form ammonia from protein amide nitrogen does not constitute evidence that it cannot also form ammonia from urea.

Indeed, there is now direct evidence in favor of the view that the urinary ammonia arises from urea. Thus, Mann and Bollman (10), in a recent paper on ammonia formation following complete removal of liver, make the following statement.

"The cessation of the formation of urea in dogs following complete removal of the liver provides a means of studying the formation of ammonia. Following hepatectomy the urea in the blood and tissues decreases progressively, and at the same time its excretion in the urine decreases markedly. As the body becomes depleted of preformed urea, its concentration in the urine becomes extremely low. The ammonia excreted after hepatectomy is equivalent to that excreted by similarly treated normal animals, and it would appear that the amount is related to the acid-base equilibrium of the animals. However, when the urine of the dehepatized dog becomes extremely low in urea, it is always found that the ammonia excretion has likewise diminished. Intravenous injection of urea at this time causes a definite increase in the excretion of ammonia."

We believe that experimental data reported up to the present time warrant the following conclusions.

1. The urinary ammonia is formed by the kidney.
2. Urea is probably the precursor of the ammonia found in the urine.
3. Ammonia plays no part in neutralization of acids transported in the blood.
4. Satisfactory evidence is lacking that ammonia is utilized for the intracellular neutralization of acids within the organism.

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STUDIES ON CREATINE.

I. THE EFFECT OF CREATINE ON THE BLOOD SUGAR.*

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(Received for publication, March 25, 1929.)

In 1918 Watanabe (1) found that guanidine hydrochloride given to rabbits will reduce the blood sugar. Recently, Frank (2) and his associates have prepared several derivatives of guanidine with the hope of finding one which would be little toxic and at the same time would retain the power to reduce the blood sugar. They believe they have found such a compound in synthalin, diguanidyldodecane. They offer this compound as a substitute for part of the insulin in the treatment of diabetes. Blatherwick, Sahyun, and Hill (3), however, believe that the action of synthalin may be due to a toxic effect on the liver. With these experiments in mind we have given creatine to dogs and have followed the blood sugar changes. Since creatine is a guanidine compound normally present in the animal body, it was believed that it could be introduced into the animal body without toxic effect.

EXPERIMENTAL.

Methods.

In the study of subcutaneous administration of creatine small dogs were used which had been fasted since the previous afternoon. The experiments were begun about 9.00 a.m. A sample of blood was taken from a leg vein for sugar determination and the creatine immediately given. Other blood samples were taken at hourly intervals. The creatine was administered in water solution

* A preliminary report of part of this work was presented at the twenty-second annual meeting of the American Society of Biological Chemists at Ann Arbor, April, 1928.

whether given subcutaneously or by mouth. Blood sugar was determined by the Folin-Wu method modified for use with the Lewis-Peebles colorimeter (4). In the study of administration of creatine *per os* the procedure was otherwise the same except as indicated below. Glucose tolerance tests were made both with and without added creatine. A blood sample was taken for sugar determination, and glucose (1.75 gm. per kilo) was given immediately by stomach tube. Blood samples were then taken at hourly intervals. When creatine was given, it was added to the glucose solution at the time of administration. In some cases

TABLE I.
Effect of Subcutaneous Injection of Creatine on Blood Sugar.

Dog.	Weight.	Creatine given.*	Blood sugar, mg. per 100 cc.				
			Normal.	Hrs. after administration of creatine.			
				1	2	3	4
	<i>kg.</i>	<i>gm.</i>					
A	6.0	0.18	74	60	66	81	
B	6.0	0.18	72	56	61	81	
D	8.0	0.24	79	60	63	62	70
E	7.2	0.22	88	74	74	80	90
G	7.0	0.21	100	80	82	86	92
H	8.0	0.24	88	64	63	70	83
I	3.5	0.11	81	74	65	77	79
J	3.5	0.11	94	85	72	80	76

* These figures are approximate only. The crystallizing of the creatine on cooling made quantitative injection difficult.

the tolerance test with creatine was made first and in others the order was reversed.

Subcutaneous Injection of Creatine.

Subcutaneous injections of creatine were made in eight dogs (Table I). A fall in the blood sugar occurred in each case. The maximum effect is shown in Dog H. In this case the extreme drop is 25 mg. of glucose per 100 cc. of blood, and the sugar is somewhat low at the 4th hour. This dog proved later (Table III) to have a lowered glucose tolerance. The minimum effect is shown in Dog B. Here the drop in blood sugar is only 16 mg. per 100 cc. The

recorded amount of creatine injected, however, is only approximate since creatine is not very soluble unless the water is hot and some always crystallizes out in the syringe.

TABLE II.
Blood Sugar Changes after Oral Administration of Creatine.

Dog.	Weight.	Creatine given.	Blood sugar, mg. per 100 cc.					
			Normal.	Hrs. after administration of creatine.				
				1	2	3	4	5
	kg.	gm.						
K	6.0	1.00	96	60	62	72	77	
H	8.0	0.80	72	66	63	72	73	
L	12.5	0.313	87	78	69	74	75	
"	12.5	0.625	82	74	68	65	70	
"	12.5	1.25	88	59	71	75		
E	7.0	0.175	80	74	75	80		
"	7.0	0.35	80	66	72	82		
"	7.2	0.72	72	58	47	57	67	
G	7.0	0.70	89	77	70	81	80	
"	7.0	1.40	97	85	67	77	80	94
"	7.0	2.80	72	60	63	65	67	
"	7.1	2.84	72	65	65	68	66	
F	7.2	0.72	80	62	57	56	60	
"	7.2	1.44	78	62	56	60	60	
"	7.2	2.88	90	66	61	59	61	70
"*	6.5	0.65	53	53	52	54		
M	8.0	0.20	86	73	71	66	77	
"	8.0	0.40	87	77	71	75	73	
"	8.0	0.80	86	60	69	68	81	
"*	7.0	0.70	50	(54†) 52	51			

* Fasted 3 days.

† Sample taken $\frac{1}{2}$ hour after creatine administration.

Oral Administration of Creatine.

The effect on the blood sugar of oral administration of creatine is shown in Table II. Six dogs were fed creatine, 100 mg. per

kilo. The fall in the blood sugar was fairly constant, 19 to 29 mg. per 100 cc. of blood, except in the case of Dog H which had the comparatively low initial value of 72 mg. per 100 cc. The amount of food taken by these dogs during the few days before the experi-

TABLE III.

Effect of Oral Administration of Creatine on Blood Sugar Curve Following Ingestion of Glucose.

Dog.	Weight.	Glucose by mouth.	Creatine given.	Blood sugar, mg per 100 cc.				
				Normal	Hrs after administration of creatine.			
					1	2	3	4
	kg.	gm.	gm					
C	9 7	17 0	0 0	83	130	135	114	
"	9 7	17 0	1 00	76	100	101	90	
E	7 0	12 0	0 0	70	120	90	70	
"	7 0	12 0	0 70	73	88	80	62	68
"	7 0	12 0	1 40	82	96	67	60	70
"	7 0	24 0	2 80	72	89	75	58	66
F	7 2	12 2	0 0	78	150	96	81	
"	7 2	12 2	0 72	71	102	90	91	82
G	7 0	12 0	0 0	80	132	93	86	80
"	7 0	12 0	0 70	98	110	85	63	86
H	8 0	14 0	0 80	71	172	80	68	77
" *	8 0	14 0	0 0	78	236	200	160	84
" †	8 0	14 0	0 80	80	171	132	60	80
"	8 0	28 0	1 60	80	180	160	92	68
"	8 0	14 0	0 80‡	83	70	174	64	66

* Urine of 4 hour period after glucose reduced Fehling's solution.

† Urine of 4 hour period after glucose did not reduce Fehling's solution.

‡ Creatine given 1 hour before glucose.

ment was not recorded. The difference in initial blood sugar levels appears to be due to difference in food intake.

Extreme hypoglycemia accompanied by convulsions is produced by large doses of insulin or synthalin. We attempted to produce hypoglycemic convulsions by increasing the dose of creatine. Dogs F and G were fed doses of 200 and 400 mg. per kilo. There

is no material increase in the effect of creatine with the large dose. The smaller effect shown in Dog G with 400 mg. per kilo is probably due to the lower initial blood sugar value. Smaller doses (25 and 50 mg. per kilo) were given to Dogs E and L. The decrease was somewhat less marked in these cases.

Dogs M and F were fasted 3 days to lower the blood sugar. In these experiments the initial blood sugar level was lower than that produced by creatine feeding normally. Under these conditions creatine feeding caused no further drop.

Several experiments were carried out to show the effect of creatine given by mouth on the blood sugar curve after giving glucosc. The results of these experiments are recorded in Table III. In the case of Dog C where the least effect is shown, the rise in blood sugar on giving 1.75 gm. of glucose per kilo alone was 52 mg. per 100 cc. of blood. When the same amount of glucose was given with 100 mg. of creatine per kilo, the rise in blood sugar was 25 mg. per 100 cc., a difference of 27 mg. per 100 cc. Dog F shows the greatest effect with the exception of Dog H. The extreme rise in blood sugar when glucose was given without creatine in the case of Dog F was 72 mg. per 100 cc.; when creatine was given with the glucose the rise was 31 mg. per 100 cc., a difference of 41 mg. per 100 cc. Dog H showed a spontaneously lowered glucose tolerance. The fasting blood sugar level was normal but rose to 236 mg. per 100 cc. when glucose was given by mouth. The effect of creatine was greater in this case. The rise in blood sugar on giving glucose alone was 158 mg. per 100 cc. When creatine (100 mg. per kilo) was given with the glucose the rise was 101 mg. per 100 cc., a difference of 57 mg. per 100 cc. of blood. Urine collected from Dog H after feeding glucose alone gave a strong reduction with Fehling's solution. When creatine was fed with the glucose the urine did not reduce Fehling's solution.

With Dogs E and H the effect of increasing the dosage of creatine was studied. With a dose of 200 mg. of creatine per kilo plus glucose Dog E showed an extreme rise in the blood sugar of the same magnitude (14 mg. per 100 cc.) that was shown with 100 mg. per kilo. The subsequent fall, however, was to a much lower level. When the dosages of creatine and glucose were both doubled the rise was still the same with a considerable subsequent fall. We tried the effect of delaying the administration of glucose for

an hour after creatine was given (Dog H). No difference in the total rise occurred but there was a more rapid return to normal.

DISCUSSION.

Creatine, because it is a guanidine compound normally present in the body, was administered to dogs to study the effect on the blood sugar level. Since it is normally present, we hoped that it would be non-toxic. In this we were encouraged by the fact that Rose and Dimmitt (5) gave as much as 20 gm. of creatine to a man in 1 day without any apparent toxic reaction, and by the recent work of Chanutin (6) who gave 10 gm. of creatine to a man daily, for 34 days. Our experience justified the hope, in so far as the gross appearance of the animal was concerned. We have under way, however, a more thorough investigation of the question.

Creatine exercises a very constant effect in lowering the blood sugar, if the initial value is not abnormally low. Large doses (400 mg. per kilo), however, do not cause an appreciably greater fall than small ones (50 to 100 mg. per kilo). In a personal communication, S. R. Benedict suggested that the fall in the blood sugar after large doses of creatine is in part masked by the high creatine content of the blood, since creatine affects the Folin-Wu reagent. We have work in progress in which the creatine content of the blood after creatine ingestion is being determined on the same sample as the sugar. Duplicate experiments on the same dog do not always show the same fall in the blood sugar. Thus far, in each case studied the smaller decreases in the blood sugar have been accompanied by higher rises in the creatine in the blood. However, because of our experiments with the fasted dogs, we feel that a basal level does exist, below which the blood sugar will not fall after creatine ingestion. It appears that there is a basal value which varies somewhat for different dogs (Dogs E and L, Table II) below which creatine cannot force the blood sugar. This is further indicated by the experiments in Table III which show that when the blood sugar level is lowered to this "basal" level by fasting, creatine has no further effect. We wish to suggest that, since creatine is normally present, some mechanism for taking care of excesses and thus avoiding dangerous hypoglycemia may be part of the normal machinery of carbohydrate metabolism.

The question naturally arises as to whether this effect of creatine is on the sugar of the blood or on some other reducing substance present there. This is answered by the failure of Dog H (Table III) to excrete sugar after glucose feeding with creatine, though large quantities were excreted when glucose alone was given. This question is also answered by an experiment already described in a preliminary report (7). 98.4 gm. of glucose were taken by one of us (I.H.M.) with 50 mg. of creatine per kilo. No rise in blood sugar occurred. It seems unlikely that no rise in blood sugar would have been observed after taking so large a dose of glucose, if the effect of the creatine were on any other constituent of the blood.

SUMMARY.

1. Creatine given either subcutaneously or by mouth reduces the blood sugar of fasting dogs.
2. Creatine given with glucose in a glucose tolerance test diminishes or in some cases practically abolishes the rise in blood sugar.
3. Creatine is apparently non-toxic.
4. By administration of creatine it is impossible to depress the blood sugar to the point where convulsions occur.
5. A dog which otherwise excreted large quantities of glucose after glucose administration was "sugar-free" when creatine was given with the glucose.

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STUDIES ON CREATINE.

II. THE EFFECT OF CREATINE ADMINISTRATION UPON RABBITS.

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Since one of us (1) had demonstrated the production of hypoglycemia in dogs after creatine administration, it seemed desirable to investigate the possible toxic effects upon the organism of administered creatine. Hence the glycine method as employed by Blatherwick *et al.* (2) was used in this laboratory to follow the effects of creatine fed to rabbits. Because our experiments showed that administration of creatine to rabbits failed to produce the hypoglycemia characteristic of such treatment in dogs and man, the toxicity experiments with rabbits were soon discontinued and dogs were used thereafter. Our intention was to report the rabbit experiments along with the results on dogs (not yet completed), but the present report on rabbits was judged desirable in view of a statement in a recent paper by Bischoff *et al.* (3): "Hill has recently reported the hypoglycemic action of creatine when administered to dogs subcutaneously or by mouth. We were unable to duplicate his results with rabbits and suggest that an impurity in his creatine may have functioned as the hypoglycemic principle."

General Procedure.

The rabbits used were stock animals, full grown, and presumably healthy. Stock feed consisted of alfalfa hay, rolled barley, and water, with fresh vegetables at intervals. Animals were fasted at least 15 hours before the experiments were begun. Blood samples were collected from a marginal vein of the ear and oxalated. Folin-Wu filtrates were made for the determination of

sugar, urea nitrogen, and amino acid nitrogen by the Folin-Wu methods modified for use with the Lewis-Peebles colorimeter (4). All determinations were made at least in duplicate, except in two instances when the blood samples were too small, as noted in Table I. Fresh filtrates were used for sugar analyses. The compound used for all extended creatine experiments in this laboratory has been Eastman's No. 951, as received. The first lot was sampled, and a specimen after autoclaving and colorimetric comparison with standard creatinine indicated 100 per cent purity. Another portion upon recrystallization exhibited the same hypoglycemic action (in dogs) as the original.

Toxicity Experiments.

The procedure of Blatherwick, based upon the work of Lewis and Izume (5), was followed except for the time of sampling. After taking the initial blood specimen, an aqueous solution of glycine (1.0 gm. per kilo of body weight) was injected subcutaneously. Blood samples were collected 1½, 5, 10, and 24 hours after injection. Each sample was analyzed for sugar, urea nitrogen, and amino nitrogen. After an interval of at least 2 weeks, the above procedure was repeated, with the addition of creatine *per os* (50.0 mg. per kilo) just before the injections. According to Blatherwick the blood amino nitrogen should return practically to the initial level within 10 hours after injection of 1.0 gm. per kilo of glycine if the deamination rate is normal, and marked increase in blood urea nitrogen should not be observed in the last sample or two unless kidney function is impaired.

The results are compared in Table I. Obviously the creatine failed to prevent the rise in blood sugar per cent which followed deamination. Likewise there is no consistent evidence of toxicity. Only the urea nitrogen values with Rabbit R-3 are suggestive of retention. Rabbit Q-2 showed definite pathological symptoms before creatine was given, and the mild urea retention and decreased deamination rate were likely the result of this condition, which was not manifest objectively at the time the normal series was obtained.

TABLE I.
Changes in Glucose, Urea Nitrogen, and Amino Acid Nitrogen Concentrations in Blood of Rabbits after Injection of Glycine (Subcutaneously) with (B) and without (A) Creatine (Per Os).
 1.0 gm. of glycine was administered per kilo of body weight.
 50.0 mg. of creatine were administered per kilo of body weight.

Hrs. after injection	Blood constituents, mg. per 100 cc.															
	Glucose				Urea N.				Amino acid N.							
	0	1½	5	10	24	0	1½	5	10	24	0	1½	5	10	24	
Rabbit R-3. A*	93	93	105	87	79	20 3	23 0	30 7	29 3	23 0	9 5	21 8	14 8	10 0	8 8	
B	102	124	111	95	93	23 5	27 7	33 3	37 0	34 0	10 5	29 8	21 0	10.5	9 1	
Rabbit Q-2. A	90	106	94	82	93	26 0	26 7	26 8	31 2	25 0	10 3	23 9	16 3	10 4	7 8	
B*	97	98	111	103	123	27 7	27 3	29 3	30 7	30 0†	8 8	31 5†	21 6	15 0†	9 8	
Rabbit L-2. A*	77	101	111	99	92	30 2	28 3	32 0	30 3	30 8	9 8	26 1	22 1	15 1†	10 3	
B	92	111	110	103	103	27 5	30 3	33 3	31 0	28 3	8 4	21 3	18 4	11 3	8 9	
Rabbit R-2. A	94	106	102	96	95	22 7	23 3	28 7	30 2	27 3	8 6	19 6	17.7	13 3	8 1	
B	105	127	106	97	99	20 3	22 3	27 2	27 2	20 2	7 9	28 4	18 9	8 8	7 3	

* Very slight losses of injected liquids.

† Animal showed unmistakable pathological signs before creatine was fed; snuffles, unsteadiness.

‡ Single determinations.

TABLE II.

Effect of Various Modes of Administration of Creatine upon Blood Sugar Concentration of Fasting Rabbits.

Rabbit.	Weight.	Treatment. Aqueous solutions of:	Hrs. after initial adminis- tration.	Blood sugar.
	<i>kg.</i>			<i>mg. per 100 cc.</i>
O-1	2.5	125 mg. creatine intravenously.	0	111
			0.7	113
			1.5	119
Q-2	2.8	280 " " subcutaneously.	0	101
			0.4	112
			1.5	110
R-2	2.6	130 " " + 165 mg. NaH ₂ PO ₄ ·4H ₂ O, <i>per os</i> .	3.0	111
			0	110
			1.0	106
			2.0	100
G-1	2.9	368 mg. NaH ₂ PO ₄ <i>per os</i> .*	3.5	104
			0	114
			1.0	130*
			2.0	111
			3.5	110
	2.8	368 " NaH ₂ PO ₄ ·4H ₂ O + 145 mg. creatine, <i>per os</i> .*	24.0	114
			24.1	
			25.0	198*
			26.0	172
			0	114
R-3	2.5	140 mg. creatine + 336 mg. Na ₂ HPO ₄ ·12H ₂ O, <i>per os</i> .	1.0	130
			2.0	125
			2.1	
			3.0	108
			5.3	117
		140 mg. creatine subcutaneously.	0	100
			1.0	122
			2.0	112
			19.5	106
			19.6	
L-2	2.5	159 mg. Na ₂ HPO ₄ ·12H ₂ O + 125 mg. creatine subcutaneously.	20.5	105
			21.5	104
			0	116
			1.0	114
H-3	2.3	125 mg. creatine + 159 mg. NaH ₂ PO ₄ ·4H ₂ O, <i>per os</i> .*	2.5	119
			0	131
			0.6	143
			1.3	133

* Struggled. See foot-note 1.

Effect of Creatine and Phosphate Administration upon Blood Sugar in Rabbits.

In consideration of the influence of phosphates upon creatine hypoglycemia in dogs (to be reported later), a few attempts were made to produce lowered blood sugar values in rabbits by administration of phosphates along with creatine. These trials, with others in which creatine alone was given by different routes, are outlined in Table II. Except with Rabbits R-2 and G-1 (second experiment) there is not even a suggestion of a lowering effect. It is possible that the excitation incident to injecting or passing the stomach tube¹ produced enough rise in blood sugar concentration to mask any opposite effect of creatine.

Blatherwick *et al.* (2) observed no hypoglycemia in rabbits after synthalin, given orally. With subcutaneous and intravenous injections, results were irregular; some dosages (usually lethal) produced marked hypoglycemia.

Chanutin (private communication) observed no hypoglycemia in rats after creatine administration. Allen (6) stated: "The depression of hyperglycemia by myrtilin has not been demonstrable in rats or rabbits, and it has not been possible up to the present to explain the differences between species by differences in diet." The differences noted seem to indicate that the effect of creatine upon the blood sugar level is not the same in herbivorous animals (rats under laboratory conditions, and rabbits) as in carnivorous or omnivorous animals.

Without suggesting a final explanation, reference is made to the fact that the reaction of rabbits to endocrine experimentation is notoriously difficult to predict from results with other animals. A possible cause of the failure of creatine to produce hypoglycemia in rabbits is being studied in this laboratory.

¹ Because of the ease with which the blood sugar level of the rabbits is raised by excitement, great care must be used in forcible feeding when the blood sugar is to be determined in animals which have not been used often enough to become accustomed to the stomach tube. In our later experiments a small, short stem glass funnel was substituted for the stomach tube. Upon *slow trickling* of the fluid to be fed into the nearly horizontal side of the funnel, the rabbits swallowed without strangulation. This method has been used repeatedly with good results.

SUMMARY.

1. Tests of the possible toxicity of creatine upon a few rabbits by the glycine method indicated no liver injury which could be ascribed to the creatine, and, except in the case of one animal, no significant urea retention.

2. Creatine given orally, or injected subcutaneously or intravenously, alone or with phosphate administration, failed to produce hypoglycemia in rabbits in doses which (per unit of body weight) caused a definite effect in dogs.

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THE OXIDATION OF DIXANTHYDRYL UREA BY MEANS OF THE DICHROMATE REACTION.

A NEW METHOD FOR DETERMINING UREA.

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One of us described recently a method for the estimation of urea, which consisted in principle in the oxidation of its xanthidrol derivative with potassium permanganate (1). There are three features of this method which we have sought to improve. The end-point of the titration (yellow to colorless) is not sufficiently sharp, the sample to be titrated may not exceed 2 mg. of the derivative, and the relationship between the permanganate and material oxidized is merely empirical. By the use of potassium dichromate in place of potassium permanganate, we have now overcome these undesirable features.

The procedure described below is based in principle on the long established method for the estimation of carbon devised first by the Rogers brothers (2) and Brunner (3) in which oxidation is effected by potassium dichromate and sulfuric acid. Instead of measuring the resulting carbon dioxide, either gravimetrically (2-4), or volumetrically (4-7), we have chosen to determine the residual chromate (8-13). This is more rapid than the estimation of carbon dioxide and when applied to the oxidation of pure substances is equally exact and significant.

Reagents.

Sodium tungstate, 10 per cent solution (14).

Sulfuric acid, 0.66 N.

Saturated solution of barium hydroxide, approximately 7 per cent $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$.

20 per cent copper sulfate solution, 200 gm. of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) dissolved in water and made up to 1000 cc.

1 per cent phenolphthalein.

Glacial acetic acid.

Solution of xanthydrol in methyl alcohol (approximately 10 per cent). Suspend 10 gm. of xanthydrol¹ in 90 gm. of absolute methyl alcohol (113 cc). Shake vigorously. After 2 days filter through dry paper into a narrow-mouthed, well stoppered, amber bottle.

Saturated solution of dixanthidryl urea in methyl alcohol. Suspend 10 to 50 mg. of dixanthidryl urea² in 1 liter of absolute methyl alcohol contained in a well stoppered bottle. Shake at intervals during several days. Filter in portions as needed.

Standardized potassium dichromate, normal solution.

Concentrated sulfuric acid, 36 N.

Standardized sodium thiosulfate, 0.1 N.

Potassium iodide, 10 per cent solution.

Starch solution as indicator.

Procedure.

Precipitation of Dixanthidryl Urea.

a. From Urine.—Dilute 1 cc. of urine in a volumetric flask to 500 cc. Transfer 5 cc. of the diluted urine to a 15 cc. centrifuge tube, add 5 cc. of glacial acetic acid,³ and 0.5 cc. of the methyl alcohol solution of xanthydrol. Mix the contents intimately, preferably by closing the tube with a paraffined cork and shaking vigorously. Allow to stand for 1 hour during which time the

¹ We have been employing a preparation of the Eastman Kodak Company.

² To 40 cc. of 0.2 per cent urea add 140 cc. of glacial acetic acid and 20 cc. of 10 per cent xanthydrol in methyl alcohol. The latter is to be added in 5 cc. portions at 5 minute intervals. 1 hour after the last addition centrifuge the mixture and wash the crystalline precipitate with 100 cc. of ethyl alcohol, and again centrifuge. Wash the residue on a suction filter with 50 cc. of ethyl alcohol and dry at 60°.

³ In some of our early work we were inconvenienced by the formation of a ring of precipitate clinging to the glass at the surface of the fluid in the tube. This can be avoided by adding the glacial acetic acid in such a way that it runs down and over the whole of the tube wall. The xanthydrol is then added and the tube gently twirled, stoppered, and shaken. This preliminary wetting with acetic acid seems to prevent subsequent ring formation.

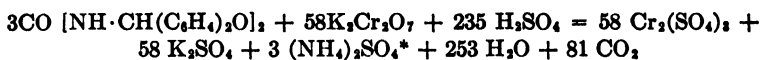
dixanthidryl urea separates slowly from solution and gathers in large loose clumps. Centrifuge for 10 minutes at about 2500 R.P.M. Decant off the supernatant fluid and wash the residue once with 10 cc. of a saturated solution of dixanthidryl urea in methyl alcohol. Again centrifuge, this time for 5 minutes, and pour off the supernatant fluid. The excess of alcohol which clings to the precipitate and the wall of the tube is removed by drying. This may be accomplished by placing in an oven for a short time at 80–90°, or by permitting the samples to stand overnight at room temperature.

b. From Blood.—Transfer 5 cc. of the Folin-Wu filtrate (14) to a 15 cc. centrifuge tube. Add 5 cc. of glacial acetic acid and 0.5 cc. of the methyl alcohol solution of xanthidrol and continue as outlined in (a) above.

c. From Muscle, Liver, and Whole Carcass.—Weigh 5 gm. of the frozen powdered tissue (15) into a large weighing bottle (40 × 80 mm.). Add 35 cc. of ice water, 5 cc. of ice-cold 10 per cent sodium tungstate, and 5 cc. of ice-cold 0.66 N sulfuric acid. Shake vigorously between additions. Permit the contents to stand with occasional shaking for 2 to 5 minutes. Filter. Transfer 15 cc. of the filtrate to a 25 cc. volumetric flask or graduated cylinder. Add 1 cc. of 20 per cent copper sulfate solution, 1 drop of 1 per cent phenolphthalein, and saturated barium hydroxide solution until the mixture just becomes alkaline. Dilute to 25 cc., shake vigorously, and filter. Place 5 cc. of the filtrate in a 15 cc. centrifuge tube and add 5 cc. of glacial acetic acid and 0.5 cc. of the methyl alcohol solution of xanthidrol. Continue as outlined in paragraph (a).

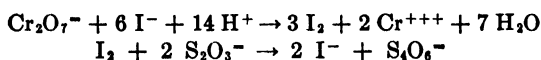
Oxidation of Derivative.

To the dry tube containing the derivative add 3.00 cc. (accurately measured) of 1.0 N potassium dichromate followed by 5 cc. of concentrated sulfuric acid. Mix intimately by means of a small rod. The heat of dilution elevates the temperature sufficiently to oxidize the derivative rapidly and completely to carbon dioxide, ammonia, and water according to the following equation.



* Cordebard (12) regards urea rather than ammonia as the end-product. His equation may be used without alteration of the relative amount of dichromate needed.

After 2 to 3 minutes wash the contents quantitatively into a 250 cc. Erlenmeyer flask, with 60 to 75 cc. of distilled water. Add 10 cc. of 10 per cent potassium iodide, and titrate with 0.1 N sodium thiosulfate in the usual fashion with a few drops of starch solution as indicator.



From the above equations it follows that 1 gm. of dixanthidryl urea requires 13.54 gm. of potassium dichromate for complete oxidation or 1 mg. requires 2.76 cc. of 0.1000 N dichromate. Since 0.1 mg. of urea is equivalent to 0.7 mg. of the derivative 1.00 cc. of 0.1000 N dichromate is equivalent to 0.0518 mg. of urea.

DISCUSSION.

Precipitation of Dixanthidryl Urea from Extracts of Muscle and Other Tissues.

In earlier papers from this laboratory reports were made of attempts to employ tungstic acid as a precipitant of muscle proteins (1, 15). While the tungstic acid extracts of blood, liver, and fetus served well for the estimation of urea by the xanthidryl method, similar extracts of muscle gave impure preparations of dixanthidryl urea. The use of Tanret's reagent in the analysis of muscle was therefore recommended. We have nevertheless been loath to complicate the system of tissue analysis by the use of more than one precipitant. It is obviously somewhat of a disadvantage when determinations of amino acid nitrogen and urea are desired in muscle to be compelled to weigh out two samples. Sometimes too the second sample can barely be spared. By treatment of the tungstic acid extract of muscle with copper sulfate and barium hydroxide we now find that the resultant filtrate is admirably adapted for the estimation of urea.⁴ The

⁴ If the estimation of amino acid nitrogen is desired the untreated tungstic acid extract is to be employed.

derivative obtained is beautifully crystalline and, as far as we can determine, free of all impurities. This procedure is based upon the well known use of copper sulfate and calcium hydroxide by Van Slyke (16) and Friedemann, Cotonio, and Shaffer (17). We have employed barium hydroxide in place of calcium hydroxide because of its greater efficacy in removal of excess sulfate, although calcium hydroxide would probably prove satisfactory. That this procedure is accompanied by no loss of urea is demonstrated by several facts: (a) Added urea is quantitatively recovered. (b) Urea values are independent of the amount of copper sulfate employed per unit weight of tissue. (c) The values obtained are identical with those yielded by extracts from two other methods of precipitation and clarification; *viz.*, Tanret's and phosphotungstic acid.⁵

We now apply this clarification procedure to tungstic acid extracts both of muscle and the whole carcass.

Oxidation of Derivative.

Boivin (18) and Cordebard (12) have already recommended the use of potassium dichromate and sulfuric acid in the estimation of dioxanthryl urea. The former estimates the carbon dioxide by the classical micro method of Nicloux (6). This is exact but unfortunately too laborious. Cordebard determines the residual dichromate but by a procedure which requires samples of urea far larger (5 mg.) than we have been able to spare.

It is fortunate that dioxanthryl urea is readily oxidized. This fact has permitted us to dispense with silver chromate as an auxiliary oxidizing agent. The inclusion of this salt in the diges-

⁵ Of many methods which we examined for the precipitation of the tissue proteins, the following were found unsuitable under the conditions of the experiments: metaphosphoric acid, uranium acetate, copper sulfate and baryta (without the preliminary use of tungstic acid), zinc chloride in ethyl alcohol, zinc chloride in methyl alcohol, tungstic acid followed by infusorial earth. The following precipitants gave filtrates which were excellent for the determination of urea by xanthidrol; potassium mercuric iodide in acetic acid (Tanret's reagent), phosphotungstic acid in 5 per cent sulfuric acid, filtration after 1 hour, the filtrate neutralized with baryta, adjusted to volume, and refiltered. Eventually, however, we found that clarification of the tungstic acid extract with copper sulfate and baryta was most economical of time and material.

tion mixture was first recommended by Simon (19) to insure complete oxidation and has since been employed by Nicloux (6), Bloor (11), and others. Apparently it is indispensable if the substance to be oxidized is a potential precursor of acetic acid in virtue of the presence of a $\text{CH}_2\cdot\text{CH}$ group (5, 13, 19). Dixanthhydril urea does not contain this grouping. We soon found indeed that the silver salt caused titration difficulties and irregular results that compelled its abandonment. Boivin had a similar experience. Also because of the ease of oxidation of dixanthhydril urea we have found that 2 to 3 minutes after addition of the sulfuric acid suffices, without further heating, to complete the oxidation. Prolonged heating is thus avoided and the whole operation facilitated.

EXPERIMENTAL.

Oxidation of Pure Dixanthhydril Urea.

100 mg. of the derivative were weighed out and dissolved in 1 liter of concentrated sulfuric acid. 1, 2, 3, and 5 cc. portions of the solution were accurately measured out and made up to 5 cc. each with concentrated sulfuric acid. 3 cc. of normal potassium dichromate were added to each. The residual dichromate was titrated with 0.1 N sodium thiosulfate. The titration values agreed with the theoretical to within 2 per cent.

Estimation of Urea in Aqueous Solution.

An aqueous solution of urea containing 100 mg. of urea per liter was prepared. Portions of the solution contained in 15 cc. centrifuge tubes were made up to 5 cc. with water. The urea was precipitated as dixanthhydril urea by the addition of 5 cc. of glacial acetic acid and 0.5 cc. of the methyl alcohol solution of xanthhydrol. The washed and dried precipitates were estimated by oxidation with dichromate according to the procedure already described. The results are presented in Table I.

Estimation of Urea in Blood and Recovery of Added Urea.

To eight 5 cc. portions of blood from a normal rabbit were added respectively 0, 5, 10, 15, 20, 25, 30, and 35 cc. of an aqueous solution of urea (0.15 mg. per cc.); 35, 30, 25, 20, 15, 10, 5, 0 cc. of

water; and to each, 5 cc. of 10 per cent sodium tungstate and 5 cc. of 0.66 N sulfuric acid. 5 cc. portions of each of the filtrates were then treated with glacial acetic acid and xanthidrol in

TABLE I.
Estimation of Urea in Aqueous Solution.

Urea solution.	0 1000 N Cr_2O_7 .	Urea found	Urea calculated
cc.	cc	mg	mg
1	1 95	0 101	0 100
1	1 96	0 102	0 100
1	1 91	0 099	0 100
2	3 86	0 200	0 200
2	3 91	0 202	0 200
2	3 88	0 201	0 200
3	5 80	0 301	0 300
3	5 83	0 302	0 300
3	5 79	0 300	0 300
5	9 69	0 502	0 500
5	9 59	0 497	0 500
5	9 67	0 501	0 500

TABLE II.
Recovery of Urea Added to Blood

Sample No	Urea found.	Urea calculated.
	mg	mg.
1	0 113	
2	0 189	0 188
3	0 268	0 263
4	0 351	0 338
5	0 429	0 413
6	0 505	0 488
7	0 576	0 553
8	0 644	0 628

methyl alcohol according to the described procedure. Analysis of the precipitates of dioxanthidryl urea gave the results presented in Table II.

Estimation of Urea in Tissues (Whole Animal) and Recovery of Added Urea.

An adult female rat after 24 hours fasting was killed, minced, and frozen according to the customary procedure. To eight 5 gm. samples were added respectively 0, 5, 10, 15, 20, 25, 30, and

TABLE III.
Recovery of Urea Added to Tissue (Whole Carcass).

Sample No.	Urea found.	Urea calculated.
	<i>mg.</i>	<i>mg.</i>
1	0.094	
2	0.131	0.131
3	0.176	0.169
4	0.209	0.207
5	0.250	0.244
6	0.296	0.292
7	0.332	0.339
8	0.367	0.376

TABLE IV.
Treatment of Urea Solutions with Copper Sulfate and Baryta.

Sample No.	20 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.	Urea found.	Urea calculated.
	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>
1	0	0.102	0.100
1 a	1	0.102	0.100
1 b	2	0.102	0.100
1 c	3	0.101	0.100
2	0	0.249	0.250
2 a	1	0.250	0.250
2 b	2	0.243	0.250
2 c	3	0.249	0.250

35 cc. of an aqueous solution of urea (0.125 mg. per cc.); 35, 30, 25, 20, 15, 10, 5 and 0 cc. of water; and to each, 5 cc. of 10 per cent sodium tungstate solution and 5 cc. of 0.66 N sulfuric acid. The filtrates were clarified with copper sulfate and baryta as described. Urea estimations were made upon 5 cc. portions of the resultant filtrates. The results are presented in Table III.

Urea Values Are Independent of Amount of Copper Sulfate Employed per Unit Weight of Tissue.

Four 2 cc. and four 5 cc. portions of an aqueous solution of urea (0.25 mg. per cc.) were diluted with water to 15 cc. To each, copper sulfate solution was added in the quantities indicated in Table IV. Phenolphthalein and barium hydroxide were added as described (warm saturated baryta was used). Each was diluted to 25 cc. and filtered. Urea was estimated in 5 cc. portions of the filtrates by oxidation of the xanthidrol derivatives. The results are presented in Table IV.

SUMMARY.

A micro method is described for the precipitation of urea as dioxanthidryl urea from urine, blood, and animal tissues. The derivative is estimated by oxidation with potassium dichromate and sulfuric acid. The excess of the oxidizing agent is determined iodometrically. For the determination of urea in muscle and other tissues the use of copper sulfate and baryta is recommended for clarification of the tungstic acid extract.

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THE BLOOD CHEMISTRY OF TWO SPECIES OF RATTLE-SNAKES, *CROTALUS ATROX* AND *CROTALUS OREGONUS*.

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During the past year the opportunity of studying the blood composition of a number of rattlesnakes presented itself. Since there are on record few, if any, reports of the chemistry of snake blood, we felt drawn to take advantage of the opportunity. The animals used were of two species, *Crotalus atrox*, the common desert rattlesnake of Arizona, Texas, and New Mexico, and *Crotalus oregonus*, the common species of the Pacific States. Of the former, five large specimens from Texas 5.5 to 6.5 feet in length were employed. The latter were obtained locally. Most of them came from Waddell Creek and Spring Valley in Santa Cruz and San Mateo Counties, and were captured as needed. None of the animals would eat voluntarily in captivity so forced feeding was resorted to. The large snakes were fed once in 2 or 3 weeks. Each animal received a ground rat weighing about 200 gm. which was administered orally by use of a long glass tube and plunger. In the earlier work eggs or blood, administered by stomach tube, were given. The Pacific rattlers which were smaller, being 2 to 3 feet in length, were not fed. They were bled but once and were used within 3 days to 4 weeks of capture.

From *Crotalus atrox* blood samples of 5 to 10 cc. were drawn by heart puncture. By the same technique, samples of 1 to 3 cc. were drawn from *Crotalus oregonus*. A period of at least 5 days after feeding was permitted to elapse before sampling. No animal was bled more frequently than once a week.

As the animals were also used for the collection of venom none was defanged. The large specimens were handled by a noose (1), and securely strapped in a trough for feeding and the collection of blood.

TABLE I.
Blood Composition.

	Human blood.	<i>Crotalus atroz.</i>	<i>Crotalus oregonus.</i>
	mg. per cent 25-35	mg. per cent 23.4 20.5 18.8 25.3	mg. per cent
Non-protein N.			
Urea N.	12-15	0 0-1.7 0.5-1.5 0-0.7	0
Amino acid N.	6-8	14.7 17.9 15.9 15.8	21.3 25.9
Uric acid.	0.7-1.5	1.9 1.2 1.2 2.3 2.6	1.5
Ergothioneine.	2.0-2.8	3.3 3.4 2.6 4.7 2.3	3.3
Creatine.	3-7	4.7 4.8	5.3
Creatinine.	1-2	1.1 1.3	1.2
Chlorides (as NaCl).	480-510	780 783 791	
Inorganic P.	3-4	5.2	10.0 10.7
Lipoid P.	12-14	19. 14 11 17	

TABLE I—*Concluded.*

	Human blood.	<i>Crotalus atrox.</i>	<i>Crotalus oregonus.</i>
	mg. per cent	mg. per cent	mg. per cent
Cholesterol.	160-190	99 101	172
Reducing sugar (as glucose).	90-100	50 67 68 54	48 48

The following analytical methods were employed.

Non-protein nitrogen, digestion of the Folin-Wu filtrate with Chiles' reagent (2), distillation of the ammonia and Nesslerization; urea, Folin and Wu (3), Luck (4), and Van Slyke and Cullen (5); amino nitrogen, Folin (6); uric acid, Benedict's indirect method (7); ergothioneine, the difference between the direct and indirect uric acid values obtained by the methods of Benedict (7); creatine and creatinine, Folin and Wu (3); chlorides, Short and Gellis (8); inorganic phosphorus, Fiske and Subbarow (9); lipid phosphorus, Whitehorn (10); cholesterol, Myers and Wardell (11); reducing sugar, Benedict (12) and Folin (13). Whole blood or protein-free filtrates thereof were used in all cases.

The results of these analyses are presented in Table I. In the first column we have reproduced for comparison, the generally accepted values for normal human blood. Although these have been drawn from the literature we ran preliminary determinations upon mammalian blood (rat, rabbit, or human) in order to satisfy ourselves of the validity of the analytical methods employed. The values tabulated for any given constituent were all obtained from different snakes. Thus the four non-protein nitrogen estimations reported for *Crotalus atrox* were made upon four different specimens. In most cases each value thus presented is the average of duplicate determinations.

DISCUSSION.

One of the most striking facts about the composition of *Crotalus* blood, which may be true of snakes generally, is its low urea

content. The method of Luck (4) yielded no visible precipitate of dioxanthidryl urea while the values obtained by the methods of Van Slyke and Cullen (5) and Folin and Wu (3) were low enough to fall possibly within the limits of experimental error. This is also true of alligator blood (14). For comparative purposes urea was determined in another reptile, the lizard, *Sceloporus occidentalis*. Owing to its small size, the whole animal rather than the blood only had to be used. Two specimens analyzed for urea by the method of Allen and Luck (15), gave values for the whole carcass of 4.2 and 1.7 mg. of urea nitrogen per 100 gm. of tissue. The presence of such a small quantity of urea is almost certainly associated with the fact that uric acid rather than urea is the end-product of nitrogen metabolism in reptiles, although this cannot be the only factor involved, for avian blood contains relatively much urea (16, 17). While in the reptile and bird uric acid appears to possess the significance which attaches to urea in the mammal, and hence is formed in relatively large quantities, the reptilian kidney succeeds in maintaining the uric acid content of the blood at a level little greater than in the mammal. The uric acid concentration in *Crotalus* blood as determined by direct or indirect methods is but slightly larger than that of mammalian blood.

The high amino acid content of *Crotalus* blood is probably associated with the possession by the animal of nucleated erythrocytes (18). Bird blood is likewise rich in amino acids.

We are at a loss to explain the high chloride, high inorganic phosphorus, and low cholesterol values in *Crotalus atrox*, especially in view of the much greater phosphate and cholesterol content of the blood of *Crotalus oregonus*.

In both species the blood sugar is very low.

SUMMARY.

Blood analyses were made upon two species of rattlesnakes, *Crotalus atrox* and *Crotalus oregonus*.

Compared to mammals, the bloods of both species contain only traces of urea. Amino acids and inorganic phosphates are present in greater quantities than in mammalian blood. Reducing sugar is low in both species. Cholesterol is low in the blood of *Crotalus atrox*. Chlorides are high.

The non-protein nitrogen, creatine, creatinine, and lipid phosphorus values are of the same magnitude in *Crotalus* and mammalian blood.

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STUDIES ON THE COMBINATION BETWEEN CERTAIN BASIC DYES AND PROTEINS.*

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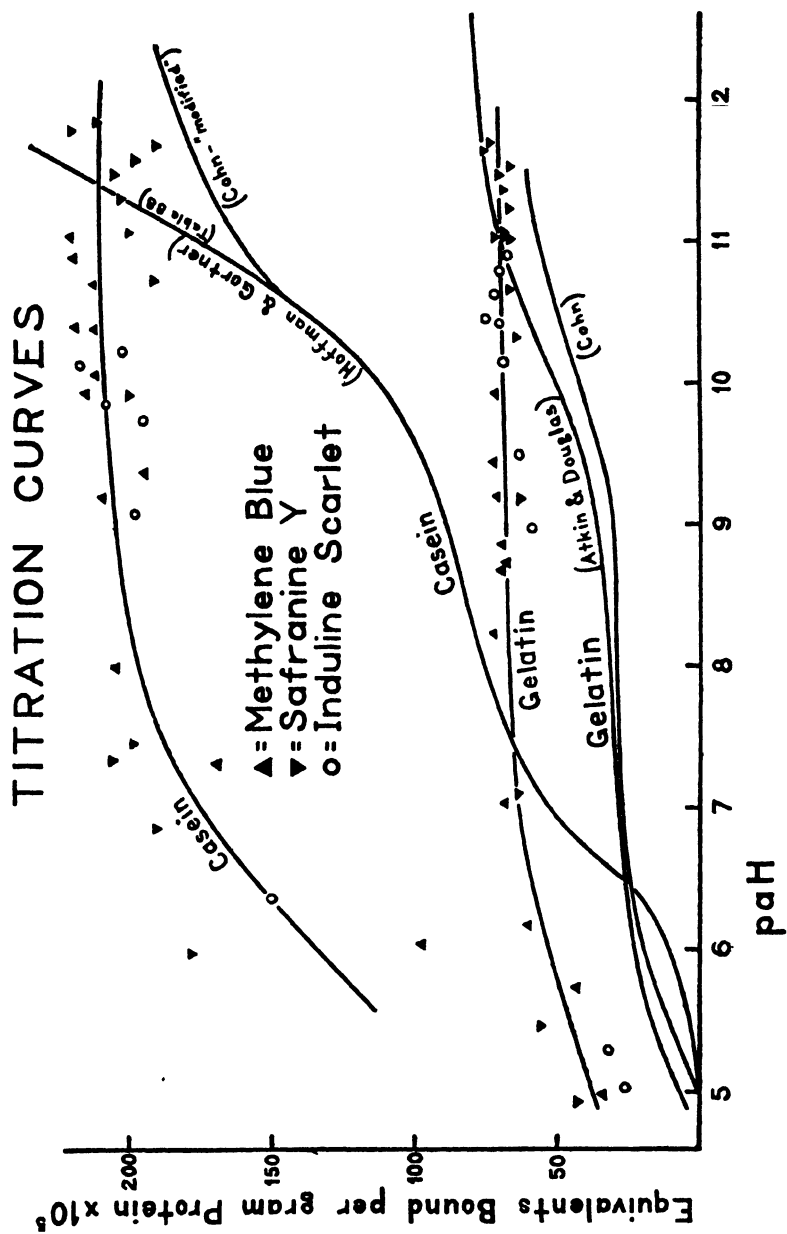
In a previous paper, Chapman, Greenberg, and Schmidt (2) reported studies on the nature of the combination which takes place between certain acid dyes and proteins. They concluded that the combination takes place in stoichiometric proportions. They also found evidence to favor the idea that the capacity of certain proteins can, within limits, be correlated with their content of basic groups. Gortner (5) has taken exception to our interpretation of the data presented and considers that they offer very striking substantiation of the conclusions reached by Hoffman and Gortner (9). While opportunity was offered to us to reply to Gortner's criticism, we preferred to allow the matter to rest on the basis of the data and conclusions originally presented. A reply would necessitate the opening of the question as to the exact definition of adsorption. The term adsorption has been generally applied to any and all reactions between a colloid and any other substance. Even the term colloid is an arbitrary one (7). Certainly the definition of adsorption is not less arbitrary. The term as stated by Robertson (13) "implies a uniformity where no uniformity exists and is, moreover, devoid of utility or meaning unless we attach to the definition some distinct idea of the nature of the underlying forces which condition the union, whether these forces be regarded as consisting of chemical (*i.e.* atomic) attractions or of capillary (*i.e.* molecular) attractions. But in forming such concrete ideas we are simply returning to conceptions which are already familiar to us in the 'crystalloid' field of chemistry and which call for no definitions which we do not already possess as the result of our general acquaintance

* Aided by a grant from the Sigma Xi society.

with the physical and chemical phenomena which are displayed by simpler and hitherto more familiar substances."

The definition of adsorption will not be attempted here. The writers believe that they and others have found sufficient evidence to place the reaction between proteins and acids or bases (*i.e.* acid or basic dyes) in the category of true chemical reactions. If the term adsorption is to be applied to this phenomenon, it will seemingly necessitate the inclusion of chemical reactions in the definition of adsorption. Such an inclusion will probably not be generally acceptable. We do not stand alone in our contentions that in dilute solutions the charges on a protein molecule are due to free ionizable groups. Simms (14) has recently stated in unqualified terms his similar belief.

The present investigation concerns itself with the combination which takes place between certain basic dyes and proteins and is a continuation of the work previously reported by Chapman, Greenberg, and Schmidt (2). They found that in order to obtain the maximum combining capacity of the proteins with certain acid dyes, it was necessary that the acidity of the solution be high. The maximum combination between protein and basic dye takes place in solutions of high alkalinity. In order to meet the experimental requirements, the basic dye must be soluble in solutions of high alkalinity and when added to the protein solutions must yield an insoluble precipitate. Many basic dyes are not soluble in solutions of even moderate alkalinity, while a number of others which are soluble do not yield insoluble precipitates with the available proteins. We were limited in our choice of dyes to methylene blue, safranin Y, and induline scarlet. These are soluble in solutions of moderate alkalinity. Before these dyes could be used it was first necessary to determine the pH range over which there was no danger of the dye becoming insoluble. Various amounts of 0.1 N sodium hydroxide were added to the neutral dye and the solution was filtered to remove any precipitated dye base. The filtrate was added to the protein solution. If a precipitate resulted, that concentration of alkali was considered suitable for the particular dye and the protein, since the protein-dye precipitate was thus shown to be more insoluble than the basic dye. The titrations were made slowly and with rapid stirring in order to prevent any precipitation of dye; or if dye did



Fig

precipitate sufficient time was given to effect resolution. In all cases a margin of safety to prevent precipitation of dye by high alkalinity was observed. It was found possible to titrate gelatin with safranin to p_{a_H} 11.8, with methylene blue to p_{a_H} 10, and with induline scarlet to p_{a_H} 11. It was found necessary to remove the protein-dye precipitate by centrifuging instead of by precipitation in order to prevent the loss of dye by filter paper adhesion. The proteins were brought into solution and the desired p_{a_H} was obtained by the addition of sodium hydroxide. In other respects the technique was the same as that which has been described by Chapman, Greenberg, and Schmidt (2).

Methylene blue and safranin Y were obtained from Coleman and Bell and induline scarlet was obtained from the General Dyestuff Corporation. The latter company discontinued the manufacture of induline scarlet after the present work was begun, and we were unable to obtain sufficient dye to complete our studies. Hence this dye was not used for the titration of edestin. The preparation of the proteins which were used has been described by Chapman, Greenberg, and Schmidt (2). They were used in approximately the same dilutions as stated by them.

The gelatin solution was prepared by dissolving 2 gm. of isoelectric gelatin in 1 liter of distilled water. It was permitted to stand in the ice chest overnight. Casein, edestin, and fibrin were each dissolved by addition of minimum amounts of alkali in order to effect solution and at the same time to keep the alkalinity at a low level. The solutions were filtered and permitted to stand in the ice chest overnight.

Gelatin and casein were titrated respectively with each of the three dyes at varying hydroxyl ion concentrations. The results of these titrations are shown in graphical form in Fig. 1. In the same figure we have included two basic titration curves of casein, one taken from Cohn (3), which represents data obtained by a number of investigators, and the other taken from the paper of Hoffman and Gortner (9). Likewise two basic titration curves for gelatin are included, one the composite curve which was plotted by Cohn (3) and the other representing the work of Atkin and Douglas (1). Fibrin was titrated with each of the three dyes and edestin with two. The results are shown graphically in Fig. 2. For comparison the basic titration

TITRATION CURVES

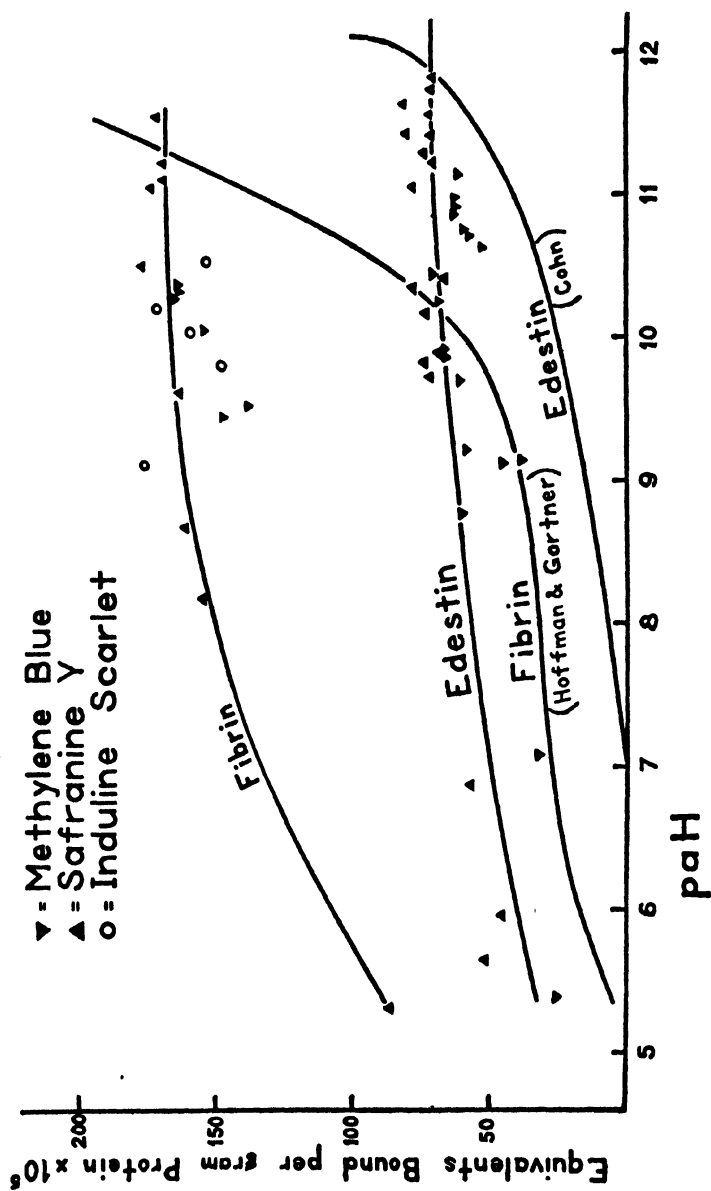


FIG. 2.

curve of edestin compiled by Cohn (3) and the basic titration curve of fibrin obtained by Hoffman and Gortner (9) have also been included. Fewer titration curves for these proteins are available than for casein and gelatin.

It will be noted that although the experimental error is considerable, the titration values for a particular protein with each of the three dyes agree fairly well. In each case the amount of dye which is taken up by the protein increases with increasing alkalinity up to a certain point. The curves are steep at first and later appear to approach asymptotically limiting values. Owing to the insolubility of the dyes at alkalinities higher than those given, it was not found possible to complete the titration curves. The dye titration curves differ in shape from those which were obtained by measuring changes in hydrogen ion activity. This is not unexpected. The latter represents an equilibrium between soluble components, while the former, owing to the insolubility of the protein dye compound, represents a reaction which is practically complete.

It has been shown that the base-combining power of proteins can, within limits, be correlated with the content of free acidic groups (4, 6, 14, 15). Such correlations are at times merely approximations, since the uncertainty in the values for the amino acid content is greater than the titration error. In many instances the data relating to amino acid content are too scant to permit even a rough correlation. The dye titration curve of gelatin shows that about 70×10^{-5} equivalents of dye are bound per gm. of protein at pH 11.8. Simms (14) found practically the same value. Hitchcock's value recalculated by Cohn (3) is 56×10^{-5} . Greenberg and Schmidt (6) found 60×10^{-5} . On the basis of the content of dicarboxylic amino acids, the expected value is 65×10^{-5} . If the value for amide nitrogen is subtracted, the value falls to 42×10^{-5} . The tyrosine content of gelatin is too small to be a significant factor in its base-binding capacity. The lack of correlation appears to indicate that at high alkalinities the acid amide group may be largely hydrolyzed. The time consumed in carrying out the titrations which may at times require several hours points to this possibility.

The dye titration curve of casein indicates that 210×10^{-5}

equivalents of dye are bound at p_{a_H} 12.0. Cohn and Berggren (4) found 183×10^{-5} for the base-combining capacity of "Hammarsten" casein. Greenberg and Schmidt (6) calculated that casein should bind 158×10^{-5} equivalents of base and by experiment obtained 160×10^{-5} . The discrepancies in the figures obtained by different workers, as summarized by Cohn (3), may be due either to opening of internal anhydrides or splitting off of amide groups. If the latter reaction takes place, it cannot be complete, since this would raise the base-combining power to 265×10^{-5} equivalents. The large error in estimating hydrogen ion activity in regions of high alkalinity is also a factor which contributes to discrepancies in data deduced from titration curves.

The dye titration curve of edestin shows that 70×10^{-5} equivalents are bound at p_{a_H} 11.5. Kodama (10) found 75×10^{-5} mols, while Hitchcock (8) reports a value in the neighborhood of 90×10^{-5} mols per gm. On the basis of the content of glutamic acid, aspartic acid, and tyrosine less the amide nitrogen for edestin given by Mitchell and Hamilton (12), the base-binding capacity should be 98×10^{-5} equivalents. With the figures for edestin given by Mendel (11), the base-binding capacity should be 38×10^{-5} (15). The dye titration curve of fibrin shows that at p_{a_H} 11.5 each gm. binds 168×10^{-5} equivalents of base. In magnitude this value is in approximate agreement with that shown by the titration curve of Hoffman and Gortner (9). No recent analytical values for the amino acid content of fibrin are available and hence no correlation with the base-binding capacity is, at the present time, possible.

SUMMARY.

1. Casein, fibrin, and gelatin were each titrated with methylene blue, safranin Y, and induline scarlet. Edestin was titrated with methylene blue and safranin Y.

2. The titration curves show that in the region of p_{a_H} 11, gelatin binds 70×10^{-5} equivalents of dye, casein 210×10^{-5} , edestin 70×10^{-5} , and fibrin 168×10^{-5} equivalents.

3. Within limits of error, and taking into account the possibility of modification of the protein taking place at high alka-

linities, a correlation between certain groups in the proteins studied and their capacity for binding base can be made.

4. This correlation suggests that the union between protein and basic dye under the experimental conditions observed takes place in stoichiometric proportions.

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THE PREVENTION OF THE TETANY OF PARATHYROID-ECTOMIZED DOGS.

III. AMMONIUM CHLORIDE.*

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In 1926 Boyd, Austin, and Ducey (1) reported that they had been able, by means of the administration of ammonium chloride, to reduce the frequency and severity of the attacks of tetany in thyroparathyroidectomized dogs. The period of survival was considerably prolonged but recovery was infrequent. Shortly afterwards, Wenner (2) described the results of his experiments in which he made similar observations but in which he had more favorable results as regards ultimate recovery. According to Wenner, the administration of ammonium chloride slightly increased the concentration of calcium in the serum, but Boyd, Austin, and Ducey did not find this always to be the case.

Ammonium chloride had previously been employed by Freudenberg and György (3) and by György (4) in the treatment of infantile tetany. Although György found that the relief from tetany was accompanied by an increased excretion of phosphorus and by an increase in the concentration of calcium in the serum, he, nevertheless, ascribed the relief from tetany to the removal of the alkalosis that he regarded as the cause of tetany.

It has been shown (5) that there is not the slightest reason for believing that there is an alkalosis in parathyroidectomized dogs. The explanation of the action of ammonium chloride

* The experimental work that forms the basis for this paper was performed at the Harriman Research Laboratory, The Roosevelt Hospital, New York. I am indebted to Mr. Joseph Gross for all of the analytical results reported here.

must be sought elsewhere than in the correction of a non-existing alkalosis.

In the experiments to be reported in the present publication, the animals employed were among those used in a study of the effects of lactose-containing diets on the symptoms following thyroparathyroidectomy (6). Circumstances beyond our control made it impossible to complete the work as it was planned, or to complete the analyses of the material that had been collected, but the results obtained seem to be of sufficient importance to warrant publication and discussion. *

The methods of analysis were, with one exception, those employed in previous experiments and described elsewhere (7). For the determination of sodium and potassium, the material was oxidized with sulfuric and nitric acids in the usual manner. The solution was then evaporated in a platinum dish and ignited. The residue was dissolved in water and treated with an excess of barium hydroxide. The combined filtrate and washings were freed of barium with carbon dioxide and the final filtrate, after the addition of a few drops of sulfuric acid, was evaporated to dryness and ignited in a platinum dish. After weighing the mixed sulfates, the potassium was precipitated as the cobalti-nitrite and weighed as such (8).

The results of the metabolism experiments and of the analyses of serum are presented in Tables I to IV. Additional data concerning diet, etc., may be found in the preceding paper of this series (6). The results of the metabolism experiments will be discussed first.

After the operation, there was a marked retention of phosphorus not accompanied by a corresponding diminution in the excretion of calcium. The significance of this finding has already been discussed (5). The excretion of sodium and potassium was followed in only one experiment, and in this there was no change in the elimination of sodium but a marked decrease in that of potassium. The amount retained was not, however, sufficiently great to combine with all the retained phosphorus. This result is in agreement with those previously obtained (9), which showed that the retention of phosphorus after parathyroidectomy is primary, and that of sodium and potassium secondary.

After the administration of NH_4Cl , the excretion of phosphorus was increased but not sufficiently to compensate for the previous retention due to the thyroparathyroidectomy so that even after 3 days of intensive treatment with NH_4Cl there still remained a net phosphorus retention. When the use of NH_4Cl

TABLE I.

Effect of Thyroparathyroidectomy and Administration of NH_4Cl upon Excretion of Ca and P and Their Concentration in Serum. Dog 72.

Date.	Daily excretion.		Analysis of serum at close of period.			Remarks.
	P	Ca	Protein.	Ca	Inorganic P.	
1928	gm.	gm.	mg. per cent	mg. per cent	mg. per cent	
Jan. 31-						
Feb. 6	0.356	0.098	4.35	9.90	6.37	Fore period.
Feb. 7	0.230	0.135				Thyroparathyroidectomy.
" 8	0.014*	0.005*	4.41	7.19	7.81	10 a.m. tetany; removed 140 cc. blood and injected 500 cc. 0.8 per cent NaCl . Tetany ceased but returned at 1 p.m. 100 cc. 5 per cent NH_4Cl by stomach tube. Tetany ceased.
Feb. 9	0.471	0.203	4.86	6.75	6.35†	150 cc. 5 per cent NH_4Cl at intervals; no tetany. Dog refused food and died during night of Feb. 12-13, apparently in tetany.
Feb. 10	0.429	0.116				

* No feces voided on this day.

† Slight hemolysis.

was discontinued, the retention of phosphorus in one dog (Dog 74) became more marked than it had originally been and subsequent resumption of the NH_4Cl treatment increased it to only about the level observed in the fore period. That the increase in the excretion of phosphorus produced by NH_4Cl is followed by a decrease after the use of NH_4Cl is discontinued was observed by Haldane, Wigglesworth, and Woodrow (10). With

a smaller dose, Bernhardt (11) observed a similar though less marked effect. It is probable that the mechanism responsible for these results in man was also involved in the greater reten-

TABLE II.
Effect of Thyroparathyroidectomy and Administration of NH_4Cl upon Excretion of Ca and P and Their Concentration in Serum. Dog 74.

Date.	Daily excretion.				Analysis of serum at close of period.				Remarks.
	P.	Ca	K	Na	Protein.	Ca	Inorganic P.	Titrateable acidity.	
1928	gm.	gm.	gm.	gm.	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
Mar. 12-18	0.483	0.216	0.698	0.211					Fore period. Thyroparathyroidectomy, Mar. 19.
" 19-20	0.266	0.224	0.465	0.205					
Mar. 21-24	0.495	0.191	1.378	0.348					Tetany Mar. 22, relieved by NH_4Cl . In all, 1400 cc. 2.5 per cent NH_4Cl in 3 days.
Mar. 25-27	0.172	0.145	0.068	0.052	5.77	6.14	5.09	33.5	Tetany at close of period.
Mar. 28-29	0.502	0.247	0.399	0.105					300 cc. 2.5 per cent NH_4Cl on each of 3 days.
Mar. 30	0.425	0.038	0.672	0.053	5.35	5.87	2.92	20.7	No feces. 300 cc. 2.5 per cent NH_4Cl .
Mar. 31-Apr. 2	No analyses.				6.05	5.17	4.87	35.8	Severe tetany at close of period.

tion of phosphorus in the after period and in the lessened effect of NH_4Cl in the second experiment upon this dog.

The action of NH_4Cl upon the excretion of calcium was not constant. In Dog 72 (Table I), in the first experiment upon Dog 74 (Table II), and in the second experiment upon Dog 77

(Table IV), the effect appears to have been negligible, but, in the first experiment upon Dog 77 and in the second upon Dog 74, there was a decided increase in the excretion of calcium. In the latter case, the increase had been preceded by an even greater retention, but this was not so in the case of Dog 77. It seems likely that these inconstant results were due to varying equilibria between the effect of the ingestion of NH_4Cl tending to increase the excretion of calcium (10-12) and that of the parathyroid insufficiency tending to diminish it.

The excretion of potassium and of sodium was increased by the ingestion of NH_4Cl and the increase was, in the first experi-

TABLE III.
Analyses of Serum of Dog 76.

Date.	Protein.	Ca	In-organic P.	Titrat-able alkali.	Remarks.
1928	per cent	mg. per cent	mg. per cent	cc. 0.1 N per 100 cc.	
Apr. 16	5.50	11.8	6.40	35.7	Before thyroparathyroidectomy.
" 19	5.03	6.4	7.33	33.0	At onset of tetany.
" 25	5.66	5.3	8.06*		After administration of NH_4Cl , Apr. 21-23, but not Apr. 24. Tetany 3 hrs. after drawing blood.

* Some hemolysis.

ment on Dog 74, much greater than the previously established retention. After the administration of NH_4Cl was discontinued, there was a marked retention of both potassium and sodium, and, when NH_4Cl was again administered, the excretion of base, though increased, did not rise to even the preoperative level. The average excretion for the entire postoperative period, *even though it closed with 3 days of NH_4Cl administration*, was less than that during the fore period. However, the retention of base was not nearly so marked as that of phosphorus. Once more, we seem to have here the results of the balance between the action of NH_4Cl increasing the excretion of base (10, 12) and that of parathyroid insufficiency diminishing it.

Examination of the results of the analyses of the sera (Tables I to IV) shows that the calcium content was not, regularly,

TABLE IV.

Effect of Thyroparathyroidectomy and Administration of NH_4Cl upon Excretion of Ca and P and Their Concentration in Serum. Dog 77.

Date.	Daily excretion.			Analysis of serum at close of period.				Remarks.
	N	P	Ca	Protein.	Ca	Inorganic P.	Titratable alkalinity.	
	gm.	gm.	gm.	per cent	mg. per 100 cc.	mg. per 100 cc.	cc. 0.1 N per 100 cc.	
1928								
Apr. 5-8	2.48	0.256	0.245					Fore period.
" 9-13	2.60	0.168	0.250					Thyroparathyroidectomy, Apr. 10. Last day of fore period included because of retention of urine.
Apr. 14-16	5.11	0.447	0.318	5.73	9.38	5.00	23.6	Tetany Apr. 14, relieved by NH_4Cl . 4 doses of 100 cc. 2.5 per cent NH_4Cl each day.
Apr. 17-22		0.170	0.236	5.88	4.91	8.56*		Faint tremor Apr. 21-22, more marked on morning of 23.
Apr. 23		0.342	0.232					3 doses of 100 cc. 2.5 per cent NH_4Cl .
Apr. 24-26		0.185	0.187	6.00	5.78	8.46	42.2	200 mg. irradiated ergosterol Apr. 24; 100 mg. each day, Apr. 25-26.
Apr. 27		0.229	0.111	5.45	4.99	9.39*	37.2	Did not eat all of food. Tetany at close of period.

* Some hemolysis.

higher when the animal was free from tetany, following the administration of NH_4Cl , than it was when the animals were in active tetany. The relief from tetany was, therefore, not

due to an increase in the concentration of *total calcium* in the plasma.

The determinations of inorganic phosphate were, unfortunately, marred by the frequent occurrence of hemolysis. Such as they are, the analyses indicate a lowered concentration after the use of NH_4Cl . But, since the concentration of inorganic phosphate in the plasma is not always increased after thyroparathyroidectomy, this diminution even if established can scarcely be the sole cause of the relief from tetany. However, whatever the situation in the plasma may be, the administration of NH_4Cl probably diminishes the concentration of phosphate in some tissue or tissues. The increase in the excretion of phosphorus was probably not derived from the bones for it was not accompanied by a corresponding increase in the excretion of calcium. It is not likely that the increase was due to augmented tissue catabolism for the increase in the excretion of nitrogen was less, and not more, than the nitrogen content of the NH_4Cl used.

The use of NH_4Cl unquestionably produced an acidosis and, as a result of this and the reduction in the concentration of phosphate, an increase in the concentration of *ionic calcium* may be assumed. A similar increase in the concentration of ionic calcium in the *plasma* may, upon the basis of similar reasoning, be assumed to have occurred in the experiments of Wilson and his associates (13) with hydrochloric acid, of Swingle (14) with uranium salts, and of Swingle, Wenner, and Stanley (15) with carbon dioxide. That such an increase may have been responsible for the relief from tetany cannot, upon the basis of present evidence, be denied. However, the effect of acidosis does not appear to be limited to the tetany of parathyroid insufficiency. Carbon dioxide has been found to inhibit the convulsions of strychnine poisoning (16) and lactic acid interferes with the action of a number of convulsive poisons (17). Even in a normal individual, the administration of ammonium chloride diminished the electric excitability (18). It may be that in all of these instances the diminished reflex excitability is due to an increase in the concentration of calcium ions but, in the absence of more direct evidence, it seems best to say merely that acidosis produces a diminution in the reflex

excitability and therefore relieves the tetany of parathyroid-ectomized dogs.

In the case of the acidosis produced by ammonium chloride, the increased excretion of phosphate, of sodium, and of potassium, and the consequent diminished concentration of all of these in certain tissues, unquestionably may contribute to the lowering of the reflex excitability.

It may not be amiss to discuss here two consequences of parathyroidectomy that have not yet been considered in terms of the author's theory of parathyroid function. These are the increased concentration of inorganic phosphate in the serum, that is frequently but not always observed,¹ and the diminished excretion of sodium and potassium. Both of these phenomena may be due to possible interference with renal function brought about by the lowered calcium concentration in the plasma. That the latter should interfere with renal function is made very likely by the work of Hamburger and his associates (19). More direct evidence, such as might be obtained by studies of the sodium and potassium excretion in thyroparathyroidectomized dogs receiving enough calcium to maintain a normal concentration in the plasma, is unquestionably desirable and it is to be hoped that it may soon be forthcoming.

SUMMARY.

As has previously been reported by others, the administration of ammonium chloride to thyroparathyroidectomized dogs was found to relieve or prevent tetany.

The significant metabolic changes brought about by such treatment seem to have been the increased excretion of phosphorus, sodium, and potassium. The excretion of calcium was only slightly, if at all, increased. The concentration of calcium in the serum was sometimes, but not always, increased and that of inorganic phosphate appears to have been diminished.

The significance of these results, as well as of the decreased

¹ For examples of unchanged or diminished inorganic phosphate in plasma or serum in dogs in tetany see Dogs 17 and 23 (Greenwald, I., *J. Biol. Chem.*, **81**, 649 (1924)) and Dog 67 (Greenwald, I., and Gross, J., *J. Biol. Chem.*, **82**, 505 (1929)).

excretion of base and of the usually increased concentration of inorganic phosphate in the serum after parathyroidectomy, is briefly discussed.

It is suggested that acidosis diminishes reflex excitability and in that manner prevents or relieves tetany.

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HYDROLYSIS OF THE *d*-GLUCOSIDES OF *d*- AND *l*-METHYL-*n*-HEXYLCARBINOL WITH EMULSIN.

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The classical investigations of Dakin on the hydrolysis of esters with lipase and of Fischer on the action of trypsin on peptides have established the asymmetric nature of enzyme action. Fischer (1) has also recorded the following results with emulsin. *d*-Mandelamide-*d*-glucoside was unhydrolyzed after 48 hours, while under the same conditions *l*-mandelamide-*d*-glucoside was completely hydrolyzed after 20 hours. Further, both *d*-mandelonitrile-*d*-glucoside and *l*-mandelonitrile-*d*-glucoside were attacked at about the same rate and practically to the same extent. Mitchell (2) showed that emulsin hydrolyzes the *d*-glucoside of *l*-borneol 3.4 times as rapidly as the corresponding compound of *d*-borneol.

The action of emulsin on the *d*, β -glucosides of *d*- and *l*-methyl-*n*-hexylcarbinol has now been examined. These glucosides have not been prepared previously, so a quantity of the alcohol as obtained commercially was used to test the method before resolving the alcohol. The method employed was essentially that described by Koenigs and Knorr (3). Commercial methyl-*n*-hexylcarbinol is not absolutely inactive. It usually has a small levorotation. The sample used in these experiments had a rotation of $\alpha_{5461}^{20} = -0.21^\circ$ in a 1 dm. tube.

*Tetraacetyl- β -Methyl-*n*-Hexylcarbinyld-Glucoside.*

30 gm. of methyl-*n*-hexylcarbinol were dissolved in 100 cc. of anhydrous ether, and 5 gm. of acetobromoglucose (4) together with 5 gm. of freshly prepared and thoroughly dried silver carbonate were added. The mixture was shaken for 20 hours. A

second 5 gm. of acetobromoglucose and 5 gm. of silver carbonate were then put in, and the shaking was continued as before. Two further quantities of acetobromoglucose and silver carbonate were added later with further shaking, so that in all 20 gm. of acetobromoglucose and 20 gm. of silver carbonate were used. The ether was then evaporated off, and the uncombined alcohol was removed by steam distillation. The tetraacetyl compound was crystallized first from aqueous alcohol and finally from alcohol. From the latter solvent it separated in fine needles which melted at 95°.

Found. C 57.2, H 7.7.

$C_{22}H_{36}O_{10}$ requires C 57.4, H 7.8.

*β -Methyl-*n*-Hexylcarbinyl-*d*-Glucoside.*

4 gm. of tetraacetyl- β -methyl-*n*-hexylcarbinyl-*d*-glucoside were put into a stoppered bottle containing 240 cc. of water, 75 cc. of alcohol, and 16 gm. of barium hydroxide. The mixture was kept at 55–60° for 5 hours, and was frequently shaken until the whole passed into solution. The remaining barium hydroxide was precipitated by bubbling in carbon dioxide, the barium carbonate was filtered off, and the solution was evaporated to dryness. The residue was then extracted with alcohol, and the solution was again taken to dryness. The glucoside thus obtained was crystallized from anhydrous ether.

Found. C 53.9, H 9.4.

$C_{14}H_{25}O_6 \cdot H_2O$ requires C 54.2, H 9.2.

A quantity of methyl-*n*-hexylcarbinol was resolved by the method described by Pickard and Kenyon (5). The *d*-glucosides of the *d* and *l* forms of the alcohol were then prepared as described above for the racemic alcohol. For determining the rotations of the compounds 5 per cent solutions in alcohol were employed.

d-Methyl-*n*-hexylcarbinyl-*d*-glucoside gave $[\alpha]_{5461}^{17} = -37.8^\circ$.

l-Methyl-*n*-hexylcarbinyl-*d*-glucoside “ $[\alpha]_{5461}^{17} = -46.6^\circ$.

Hydrolysis Experiment.

Two 25 cc. graduated flasks were taken; in one was placed 0.1 gm. of the first glucoside and in the other an equal weight of the

second glucoside. The flasks were then put in a thermostat at 37°. The enzyme solution was prepared by mixing 0.05 gm. of emulsin with 60 cc. of water, filtering, and allowing to come to 37° in the thermostat.

The hydrolysis of the *d*-methyl-*n*-hexylcarbiny-*d*-glucoside was commenced by filling the graduated flask to the mark with the emulsin solution. 1 cc. was withdrawn at intervals and the

TABLE I.
*Rate of Hydrolysis of d- and l-Methyl-n-Hexylcarbiny-*d*-Glucosides.*

Time.	<i>A</i>	<i>c</i>	<i>K</i>
<i>d</i> -Methyl- <i>n</i> -hexylcarbiny- <i>d</i> -glucoside.			
<i>min.</i>			
18 (= <i>t_x</i>)	4.12	3.73	0.0468
33	6.00	1.85	0.0467
48	6.93	0.92	0.0470
63	7.40	0.45	0.0464
78	7.55	0.30	0.0457
91.5	7.72	0.13	
∞	7.85	Average 0.0465	
<i>l</i> -Methyl- <i>n</i> -hexylcarbiny- <i>d</i> -glucoside.			
105 (= <i>t_x</i>)	3.20	4.73	0.00552
150	4.24	3.69	0.00559
180	4.76	3.17	0.00549
278	6.10	1.83	0.00558
311	6.43	1.50	0.00550
405	7.02	0.91	
∞	7.93	Average 0.00554	

volume of 0.01 *N* sodium thiosulfate solution equivalent to the amount of glucose set free was determined by MacLean's method (6). After a few readings had been taken, the hydrolysis of the *l*-methyl-*n*-hexylcarbiny-*d*-glucoside was started, and thereafter the two experiments were carried on side by side. The results given in Table I were obtained. In each case *A* is the number of cc. of 0.01 *N* sodium thiosulfate solution equivalent to the glucose set free in 1 cc. of solution, and *c* is proportional to the concentra-

tion of unchanged glucoside present at times stated. K is calculated from the usual formula for unimolecular reactions.

$$K = \frac{2.303}{t_v - t_s} \log \frac{c_s}{c_v}$$

Hence *d*-methyl-*n*-hexylcarbiny-*d*-glucoside is hydrolyzed by emulsin 8.4 times as rapidly as *l*-methyl-*n*-hexylcarbiny-*d*-glucoside.

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A NOTE ON THE SPECIFIC ROTATORY POWER OF *d*-ARGININE.

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The optical activity of *d*-arginine salts was first measured in 1887 by Schulze and Steiger (1). Using the old Ventzke scale, they found for an aqueous solution of the hydrochloride such a value as, when converted into circular degrees, corresponds to a specific rotatory power of $+11.49^\circ$.¹ 12 years later Gulewitsch (2) reported $[\alpha]_D^{20^\circ}$ for the same salt as only $+10.70^\circ$ in water, and as $+21.25^\circ$ in the presence of an excess (6.75 to 13.5 molecules) of hydrochloric acid. Calculated for arginine itself, these values correspond respectively to $+12.93^\circ$ and $+25.70^\circ$. Gulewitsch's estimates have generally been accepted as final; but in 1922 Hunter and Morrell (3) stated that a specimen of the hydrochloride prepared by themselves gave, at 20° and with 7 molecules of excess acid, a value of $+21.95^\circ$, on which basis $[\alpha]_D^{20^\circ}$ for the base would be $+26.54^\circ$. Their conclusion, that Gulewitsch's results were too low, is supported by the more recent observations of Felix and Dirr (4), who for an aqueous solution of the hydrochloride found $[\alpha]_D^{20^\circ} = +12.12^\circ$ to 12.25° . Hunter and Morrell gave none of the details of their measurements; Felix and Dirr state only that the solution examined contained 2 per cent of the salt, in which case the angle of rotation observed must have been rather small for accurate measurement. Under these circumstances it seems worth while now to publish the actual observations, upon which Hunter and Morrell's value was originally based, as well as to

¹ Gulewitsch, misinterpreting Schulze and Steiger's Ventzke degrees as circular, calculated from their data a specific rotatory power of $+33.13^\circ$, and drew the conclusion that there are two arginines, the one of vegetable, the other of animal origin.

report the wholly confirmatory outcome of a recent repetition of their work.

Preparation of Materials.

Measurements of rotatory power have been made on three preparations obtained and purified in the manner described below.

(a) *Free Arginine*.—A solution of arginine, prepared from hydrolyzed gelatin by the well known silver-baryta method, and containing about 25 gm. of the base, was neutralized with nitric acid, treated with 25 gm. of silver nitrate, and concentrated on the water bath to a volume of about 150 cc. The crystals of acid arginine-silver nitrate obtained on cooling were twice recrystallized, the yield at each stage being increased by treatment of the mother liquor with a mixture of alcohol and ether. After this process of purification, essentially the same as that recommended and used by Gulewitsch, the arginine was precipitated again by baryta, and from the thoroughly washed precipitate of arginine-silver it was recovered in the usual way as the free base. The final solution, freed from all reagents, was evaporated to a thin syrup and allowed to crystallize in the ice chest. There were obtained thus 10.7 gm. (vacuum-dried) of perfectly white crystalline material, consisting of free arginine without any detectable admixture of carbonate. A second crop of 7.2 gm., obtained by evaporation of the mother liquor, was discarded as possibly less pure.

(b) *Arginine Hydrochloride I*.—When the preparation just described was found to have a rotatory power higher than the expected, it was at first supposed that it was contaminated by some substance having a higher rotation than arginine. For this reason further purification was sought by conversion into, and recrystallization of, the hydrochloride. The entire first crop of arginine, including that recovered by evaporation of the solution used in the polarimeter, was dissolved in water, and exactly neutralized by hydrochloric acid. The solution was evaporated to dryness, and the completely crystalline residue was twice recrystallized from the smallest possible volume of boiling 70 per cent alcohol, to which was added, as it cooled, just enough ether to produce a permanent opalescence. In this operation a slight difficulty was caused by the fact, noticed already by Gulewitsch, that at certain not com-

pletely defined concentrations of alcohol the salt becomes less soluble as the temperature rises, so that, as the boiling point is approached, it may separate from its solution in the form of oily droplets. The same phenomenon is apt to occur also upon the incautious addition, at a later stage, of too much ether. In either instance the droplets rapidly settle to the bottom in a heavy oily layer. This layer may be left undisturbed, in which case it will usually in time solidify into a mass of crystals; but it was found better to bring it, if it formed, back into solution by the addition, at the first stage, of a drop or two of water, or, at the second, of a

TABLE I.
Analysis of Preparations.

Substance analyzed.	Total N.		Amino N.	
	Found.	Calculated.	Found.	Calculated.
	per cent	per cent	per cent	per cent
Arginine.	31.90 } 31.90 } 31.90	32.18	7.99 } 8.00 } 8.17* 10.34†	8.00 8.04
Arginine Hydrochloride I.	26.53 } 26.42 } 26.45 }	26.47 26.60	6.65 } 6.71 } 7.03*	6.68 6.65

* After $\frac{1}{2}$ hour in the deaminizing chamber.

† After 3 hours in the deaminizing chamber.

sufficient quantity of hot alcohol. The complete success of the recrystallization depended then upon a proper adjustment of the concentrations of water, alcohol, and ether—an adjustment easier to attain in practice than to describe.

The product of the double recrystallization was washed with ice-cold 95 per cent alcohol and dried *in vacuo* over sulfuric acid. It then weighed 7.3 gm.

(c) *Arginine Hydrochloride II.*—This was prepared in exactly the same manner as the preceding specimen, but 7 years later and from a different lot of gelatin.

Analysis of Preparations.

In Table I are given the results of determinations of total nitrogen (Kjeldahl) and amino nitrogen (Van Slyke) carried out on the arginine preparation and on Arginine Hydrochloride I. Before analysis each preparation was dried to constant weight, the first at 110°, the other at 140°. Neither lost in drying more than 0.1 per cent of its weight, so that neither contained water of crystallization. Unless otherwise indicated in the table, deamination in the Van Slyke apparatus was allowed to proceed for 5 minutes only. This, it will be seen, yielded the theoretical amount of nitrogen for the one amino group. When the reaction was allowed to go on longer, there was a gradually increasing yield of nitrogen; but the data are far from confirming the statement of Sekine (5) that arginine loses one-half of its total nitrogen in 2 to 3 hours of treatment with nitrous acid.

Arginine Hydrochloride II was not analyzed; but its (corrected) melting point was the same (219°) as that of the earlier preparation. This melting point agrees sufficiently well with those observed by Cox (6) (222°) and by Felix and Dirr (218°), and differs, like them, from that given by Gulewitsch (208–209°). When heated above its melting point the substance solidifies again, and finally decomposes, with copious evolution of gas, at 230° (according to Felix and Dirr at 235°).

Determination of Rotatory Power.

For the determination of rotatory power a weighed quantity of the thoroughly dried substance—about 2.5 gm. in the case of arginine, and about 3 gm. in the case of the salt—was dissolved in a total volume of 25 cc., this volume including as much HCl of constant boiling point as was necessary to provide a total of 8 molecules of acid for each molecule of base. The angles of rotation were measured for the D line, three different polarimeters (by Schmidt and Haensch, Duboscq-Pellin, and Hilger respectively) being made use of from time to time. The results were checked in two ways: (1) by repeating each determination with a second polarimeter, and (2) by conducting, along with the final test, a parallel experiment upon pure glucose. In this control experiment a solution containing 5.196 gm. per 100 cc. gave, as the average of several concordant measurements, a rotation of +6.00°, cor-

responding to a specific rotatory power of $+52.50^\circ$ (theoretical for *d*-glucose, $+52.61^\circ$).

The results with the arginine preparations are given in Table II, in which *c* is the concentration, in gm. per 100 cc., of the base (in Observations 1 and 2) or the salt (in Observations 3 to 6), and the other symbols have the usual significance.

TABLE II.
Determinations of Rotatory Power.

Observation No.	Description of material.	Molecules of HCl for each molecule of arginine.	Instrument used.	<i>t</i>	<i>l</i>	<i>c</i>	α	$[\alpha]_D^{t^\circ}$ for:	
								Arginine hydrochloride.	Arginine.
				$^\circ\text{C.}$	<i>dm.</i>	<i>gm. per 100 cc.</i>	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
1	Arginine.	8	Schmidt and Haensch.	20	2.2	9.973	$+5.80$	$+21.85$	$+26.43$
2	"	8	Duboscq-Pellin.	20	2.0	9.973	$+5.30$	$+21.97$	$+26.57$
3	Arginine Hydrochloride I.	8	"	21	2.0	11.932	$+5.24$	$+21.96$	$+26.56$
4	" "	8	Schmidt and Haensch.	21	2.2	11.932	$+5.76$	$+21.95$	$+26.55$
5	Arginine Hydrochloride II.	8	" "	25	2.2	11.870	$+5.75$	$+22.02$	$+26.63$
6	" "	8	Hilger.	25	2.2	11.870	$+5.72$	$+21.90$	$+26.49$
Average								$+21.94$	$+26.54$

The observations recorded were made at temperatures varying between 20 – 25° , but, since Gulewitsch found only slight differences between 20 – 30° , the average of the six sufficiently concordant results may fairly be taken as the rotatory power at 20° . On this basis $[\alpha]_D^{20^\circ}$, in the presence of an excess of hydrochloric acid, is found to be $+26.54^\circ$ for *d*-arginine or $+21.94^\circ$ for its hydro-

chloride. These values, obtained from materials of which the analytical data attest the purity, are 3 per cent higher than Gulewitsch's. It seems fair to conclude that Gulewitsch's material was partly racemized. There is of course no guarantee that the preparations here described were themselves entirely free from the levorotatory isomer, and it is possible that perfectly pure *d*-arginine has a rotatory power even greater than that here given.

SUMMARY.

The specific rotatory power of *d*-arginine, measured for the D line at 20° and in the presence of an excess (8 molecules) of hydrochloric acid, is at least +26.54°; the corresponding value for *d*-arginine hydrochloride is +21.94°.

Treated with nitrous acid according to the technique of Van Slyke, arginine gives off in 5 minutes at room temperature exactly one-fourth of its total nitrogen. In half an hour the yield is about 5 per cent, in 3 hours about 30 per cent, greater. The statement of Sekine that the nitrogen yield is doubled in 3 hours was not confirmed.

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A STUDY OF THE ACTION OF TRYPSIN ON CASEIN.*

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If the present comparatively rough classification of protein split-products (proteoses, peptones, etc.) based on the general complexity of their molecules is assumed to be approximately correct, it becomes of interest in studying the tryptic hydrolysis of casein to determine their rate of production. It seemed then that a study in which the progress of the formation of these split-products was followed might enable one to form a clearer picture of the hydrolysis and possibly to compare the value of some of the methods heretofore applied in the estimation of trypsin.

When a solution of trypsin is added to a faintly alkaline solution of casein, the enzyme, at the start of the hydrolysis, is exerting its activity solely on the original substrate and those of the products of autohydrolysis formed in putting the substrate into solution. After the digestion commences the amino nitrogen determinable by the Van Slyke or Sørensen methods increases. It then becomes of interest to determine the relation between the production of amino nitrogen, the disappearance of original substrate, and formation of one or more classes of split-products.

To determine the proportion of proteoses at any stage of the digestion it is necessary to use salting out methods. While the separation of proteoses into deuter-, hetero-, etc., proteoses has fallen into disuse (1), the impression still persists that a complete saturation of their solution with a suitable salt will precipitate proteoses but not peptones. This led to a decision to make determinations of the nitrogen of those split-products of the tryptic hydrolysis of casein which were precipitable by completely saturating their solutions with zinc sulfate in following the course

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of such a digestion, as well as to make simultaneous determinations of amino nitrogen by the Van Slyke and Sørensen methods and of the total soluble nitrogen of the original undigested substrate (total soluble nitrogen by difference).

Procedure.

The sodium caseinate solutions were prepared of a concentration (by the method given by Sherman and Neun (2)) which produces a solution whose original hydroxyl ion concentration has been found to be conducive to active tryptic hydrolysis and which is within the range given by Robertson and Schmidt (3) as favorable to tryptic activity. The concentrations of enzyme¹ (dissolved in distilled water and filtered) used per 100 cc. of digestion mixture were 2.5, 5.0, 10.0, and 20.0 mg. The casein² used contained 122 mg. of total nitrogen per gm. (15.5 to 15.6 per cent total nitrogen after drying *in vacuo* over sulfuric acid). The digestions were carried out at 20°, 30°, and 40° for the four concentrations of enzyme for from 5 minutes to 72 hours on a single concentration of casein solution (1 gm. per 100 cc. of digestion mixture).

The enzyme action was stopped at the desired intervals by pouring the digestion mixture into 25 cc. of 20 per cent sodium sulfate (decahydrate) solution and 12.50 cc. of 0.2 N hydrochloric acid. After standing for an hour the undigested casein which had precipitated out was filtered off, washed with 10 cc. of distilled water, and subjected to a Kjeldahl determination. The completeness of the precipitation of the dissolved casein was tested by precipitating in this manner casein solutions to which distilled water instead of enzyme solution had been added to bring to volume. It was found that only 94 to 96 per cent of the original casein nitrogen was determinable in the precipitate, thus indicating that appreciable hydrolysis had resulted before any enzyme whatever had been added to the substrate solution and therefore rendering it necessary to make blank determinations in which all manipulations are the same except for the addition of enzyme. Such hydrolysis, or autohydrolysis, has been observed by others (4, 5).

¹ The trypsin used was described by the manufacturers (Digestive Ferments Company, Detroit) as possessing a proteolytic activity of at least 1:50 by the Fuld-Gross test and was made from hog pancreas, and probably contained a small amount of amylase.

² "Purified casein" of the Digestive Ferments Company.

The filtrate was divided into two portions, one of 25 per cent, the other of 75 per cent of the total volume. The smaller portion was used to determine the amino nitrogen by the Sørensen method (modified according to the technique described by Northrop (5)), the larger portion for the determination of proteose nitrogen by complete saturation with zinc sulfate in acid solution (6).

For the formol titration the 25 per cent portion of filtrate was first made slightly alkaline to neutral red and then brought back to approximately pH 7 by the addition of 0.1 N HCl to the appearance of the red color of neutral red. The neutral formol mixture was

TABLE I.
Amino Nitrogen Produced by Tryptic Hydrolysis in Digestions at 40°

Time	Method			Method			Method			Method		
	Soren- sen	Van Slyke (3 min)	Van Slyke (30 min)	Soren- sen	Van Slyke (3 min)	Van Slyke (30 min)	Soren- sen	Van Slyke (3 min)	Van Slyke (30 min)	Soren- sen	Van Slyke (3 min)	Van Slyke (30 min)
	2 5 mg trypsin			5 0 mg trypsin			10 0 mg trypsin			20 0 mg trypsin		
<i>hrs</i>												
1	1 5	1 4	1 4	3 0	2 7	2 9	6 0	5 7	6 0	11 5	11 5	12 3
2	2 7	2 8	2 8	6 1	5 6	6 0	11 8	11 4	12 1	16 0	15 7	16 8
4	5 6	5 5	5 7	10 9	10 6	11 1	15 2	15 1	16 0	19 1	18 9	19 9
8	9 7	9 7	9 9	13 8	13 9	14 7	20 3	20 4	21 3	22 7	22 3	23 8
12	11 5	11 8	12 2	17 2	17 2	18 0	22 9	23 3	24 4	24 8	24 8	26 1
24	15 1	15 3	15 7	19 3	19 4	20 4	26 3	26 4	27 5	28 3	29 0	30 3
48	17 4	17 9	18 6	21 5	21 8	22 7	28 9	28 8	29 8	31 2	31 5	32 8
72	19 6	20 4	20 9	22 3	22 4	23 5	29 5	29 5	30 3	31 9	32 4	33 6

Each figure represents the mean of three or more sets of determinations

then added; it was made by adding 1 cc. of phenolphthalein solution (1 per cent alcoholic) to 50 cc. of a 37 to 38 per cent solution of formaldehyde in water (commercial formalin) and making it just colorless with 0.1 N HCl after having first rendered it pink with alkali. The addition of the formol mixture makes the solution pink in the presence of the neutral red, owing to the hydrogen ion concentration increase following the formation of methylene amino acids. As 0.2 N NaOH is then added, this pink is changed to a bright yellow on the alkaline side of pH 7, which remains until about pH 8 is reached. Then, owing to the presence of the phenol-

phthalein, the solution begins to turn orange and then red. A satisfactory end-point to the operator's eye is first established with a known solution of a pure amino acid.

The Sørensen method measures the carboxyl groups which have an α -amino group exerting a neutralizing influence upon them and so, in turn, may measure the α -amino nitrogen, while the Van Slyke method is a measure of the amino nitrogen directly. The

TABLE II.
Nitrogen of Casein Split by Trypsin.

Time of action.	2.5 mg. enzyme.	5 0 mg. enzyme.	10.0 mg. enzyme.	20.0 mg. enzyme.				
20°								
hrs.	mg.	$k \times 10^{-4}$	mg.	$k \times 10^{-4}$	mg.	$k \times 10^{-4}$	mg.	$k \times 10^{-4}$
1	8.0	5.0	14.9	9.9	26.7	19.0	48.7	29.2
2	16.3	5.5	28.0	9.9	38.2	19.2	72.5	41.5
4	27.4	4.8	47.2	9.4	78.0	19.9	104.8	41.0
8	48.0	4.8	74.5	9.2	109.5			
12	64.6	4.9	94.0	9.8				
24	95.7	5.3						
30°								
1	15.0	10.0	28.0	19.8	46.4	36.6	79.8	83.1
2	31.6	11.4	50.0	20.2	82.5	44.2	108.0	93.0
4	52.1	10.7	80.1	20.8	111.5	55.2		
8	87.0	12.3	111.5	27.6				
12	102.0	12.4						
40°								
1	19.9	13.6	36.8	27.3	71.0	67.6	111.3	
2	34.4	12.6	68.3	31.8	106.5	87.2		
4	62.3	13.8	105.7	48.0				
8	101.6	18.4						

usual technique (7) was used for the Van Slyke determinations (3 minute deamination) and the results were found to agree well with those of the Sørensen method (Table I).

The 75 per cent portions of the filtrate were made acid (6) and completely saturated with crystalline zinc sulfate added in portions in the course of 72 to 96 hours, the mixture was filtered, and the precipitate washed with saturated zinc sulfate solution, and the

total nitrogen of the precipitate determined by the Kjeldahl method. The tabulated data are given in Tables II to IV and typical curves plotted from some (40°) of these values are shown in Figs 1 to 4.

TABLE III
Amino Nitrogen Produced by Tryptic Hydrolysis

Time of action	2.5 mg enzyme		5.0 mg enzyme		10.0 mg enzyme		20.0 mg enzyme	
20°								
hrs	mg	$k \times 10^4$	mg	$k \times 10^4$	mg	$k \times 10^4$	mg	$k \times 10^4$
1	1.01	0.61	1.76	1.1	3.02	2.0	4.08	2.5
2	1.57	0.50	2.77	0.86	5.72	1.8	7.09	2.3
4	2.90	0.42	5.07	0.77	8.30	1.3	11.34	1.7
8	4.98	0.37	8.81	0.67	10.39	0.80	16.25	1.3
12	6.04	0.31	10.54	0.54	13.68	0.72	17.20	0.93
24	8.91	0.23	13.67	0.36	17.40	0.47	21.44	0.59
48	12.94	0.17	16.60	0.22	21.10	0.29	25.19	0.35
72	16.81	0.11	17.35	0.11	23.73	0.16	27.01	0.19
30°								
1	1.32	0.81	2.54	1.5	4.05	2.5	7.43	4.5
2	2.05	0.64	4.89	1.5	7.69	2.3	11.63	3.6
4	3.93	0.62	8.07	1.3	12.17	1.9	15.14	2.4
8	6.78	0.54	11.23	1.1	16.11	1.3	20.06	1.6
12	9.36	0.48	13.66	0.72	18.07	0.98	22.52	1.2
24	13.31	0.32	17.53	0.47	22.42	0.62	27.50	0.78
48	16.81	0.22	20.28	0.28	24.06	0.33	31.11	0.45
72	18.43	0.12	22.11	0.15	24.90	0.17	31.96	0.22
40°								
1	1.68	1.0	3.10	1.9	6.08	3.8	11.53	7.3
2	3.08	0.9	6.10	1.9	11.57	3.7	15.70	5.0
4	5.76	0.9	11.25	1.8	15.02	2.4	18.86	3.1
8	9.89	0.8	13.88	1.1	20.31	1.7	22.45	1.9
12	11.50	0.6	17.24	0.9	22.82	1.3	24.47	1.4
24	16.81	0.4	19.12	0.5	26.35	0.7	28.64	0.8
48	19.76	0.3	21.10	0.3	28.86	0.4	31.66	0.5
72	20.88	0.2	22.29	0.2	29.17	0.3	32.08	0.3

It is a common characteristic for all concentrations and temperatures at which the digestion was studied that the proteose fraction, or that fraction of the tryptic digestion products of casein

precipitable by complete saturation with zinc sulfate, reaches a maximum at about the same time that the original casein substrate is entirely hydrolyzed into soluble products. It is interesting to note that at the maximum the zinc sulfate-precipitable nitrogen amounts to two-thirds to three-fourths of the entire casein nitrogen originally present. If the complete saturation with zinc

TABLE IV.
Proteose Nitrogen Produced by Tryptic Hydrolysis.

Time of action.	2.5 mg. enzyme.	5.0 mg. enzyme.	10.0 mg. enzyme.	20.0 mg. enzyme.
20°				
hrs.	mg.	mg.	mg.	mg.
1	6.0	11.1	19.9	37.3
2	10.8	16.6	32.7	53.9
4	24.4	31.2	56.9	81.5
8	37.3	55.8	80.7	62.5
12	45.8	66.7	70.6	48.7
24	68.7	74.9	53.1	30.4
30°				
1	11.3	16.1	31.3	57.0
2	20.8	29.4	56.6	76.2
4	35.8	53.0	77.7	64.4
8	53.5	69.9	61.6	42.7
12	68.8	57.3	47.2	37.2
24	55.8	44.9	30.4	26.0
40°				
1	16.3	30.4	48.2	77.8
2	29.0	47.4	70.8	59.7
4	45.6	68.4	60.1	41.1
8	69.7	65.5	43.4	30.1
12	80.5	60.3	35.8	
24	72.1	41.9		

sulfate here precipitates the larger molecular aggregates, it would be indicated that tryptic protein hydrolysis simulates other enzymic hydrolyses, *e.g.* that of starch, in that the major activity at the beginning of the digestion is first directed toward breaking the original protein aggregate into relatively large fractions and that these in turn are then further broken down.

In regard to the relation of amino nitrogen to proteose nitrogen production, it may be said that at the time that the proteose nitrogen is at a maximum the quantity of amino nitrogen lies within fairly narrow limits (11 to 14 mg.) and that this time also marks the disappearance of the original substrate. This is, then, a well

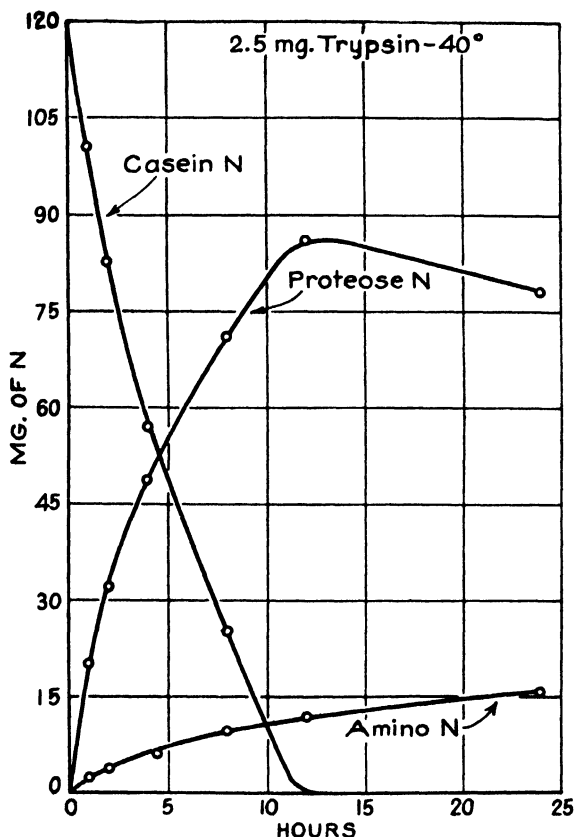


FIG. 1. Experiment with 2.5 mg. of trypsin at 40°.

marked stage in the digestion. The question of deciding on comparable conditions and quantities in determining the extent of enzyme action may be attacked from several angles. The time required to cause the disappearance of the original substrate is a gauge of activity often used. The Fuld-Gross method and a

number of similar methods for measuring peptic and tryptic activity are based on such a determination. The curves and data here shown indicate that such a method measures the time required to carry the substrate largely to the proteose stage only. In gaging the extent of hydrolysis by the amount of amino nitrogen

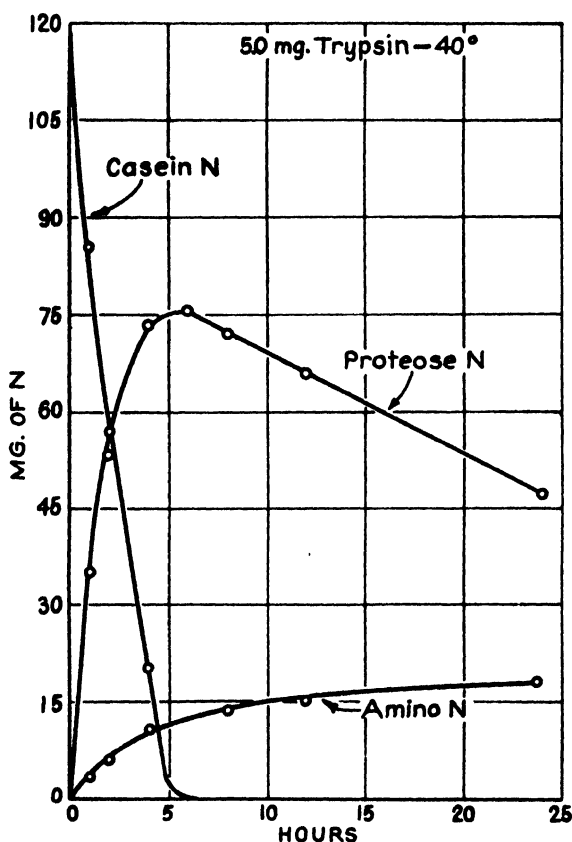


FIG. 2. Experiment with 5 mg. of trypsin at 40°.

produced, a comparison of the time intervals required to produce a given quantity of amino nitrogen has been suggested (2). The importance of choosing digestion periods of logical length in comparing the activities of two different enzyme preparations has already been indicated by work done in this laboratory. A perusal

of the data for amino nitrogen production (40°) shows that for the 1 and 2 hour intervals for the two lowest concentrations of enzyme the results are apparently irregular; that is, doubling the amount of enzyme or the time interval a little more than doubles the amount of amino nitrogen produced. Such results are quite characteristic

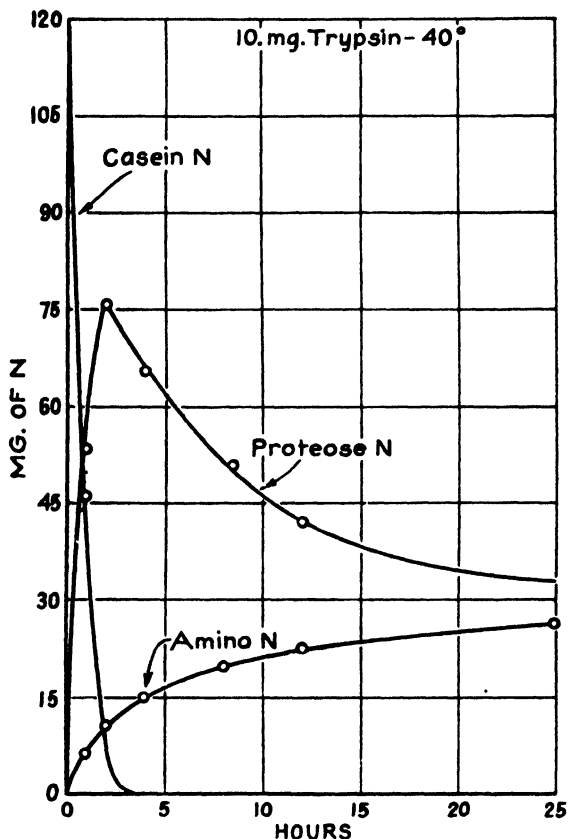


FIG. 3. Experiment with 10 mg. of trypsin at 40° .

of the short digestion periods with very small amounts of enzyme and may be explained in several ways (8). Too long digestions on the other hand reduce differences in amino, casein, and proteose nitrogen to such an extent that true comparisons are difficult or impossible. A comparison of the time intervals required to

produce 15, 30, 45, and 60 mg. of proteose nitrogen gave irregular results. A comparison of the intervals required to produce 10, 12, and 15 mg. of amino nitrogen showed that the smoothest comparisons were obtainable for the latter two quantities (amounting to 10 to 12 per cent of the total casein nitrogen) at all three tempera-

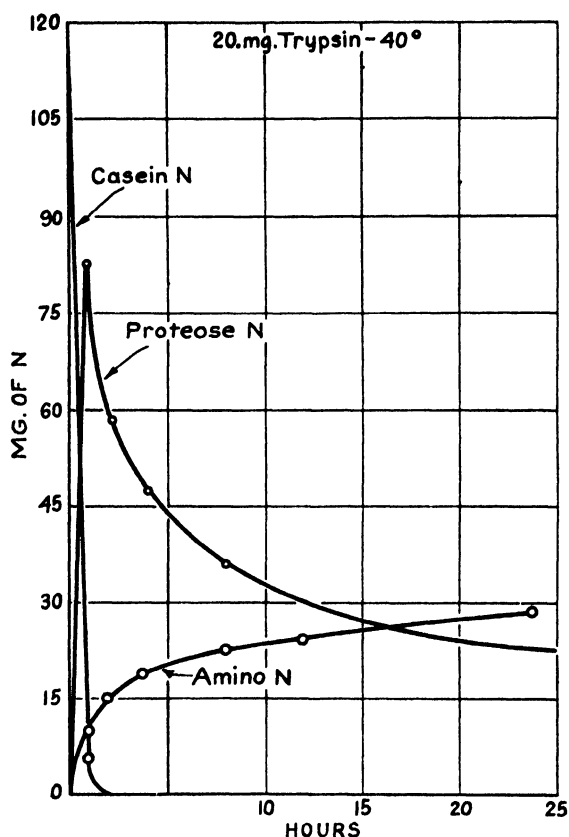


FIG. 4. Experiment with 20 mg. of trypsin at 40°.

tures. The time of disappearance of the original substrate usually lies well within this range of amino nitrogen production and may also be used as a gage of enzymic activity. These data indicate, then, that for making true comparisons of enzymic activity it is essential to choose a comparatively narrow range of amino nitro-

gen production to obtain a relationship of time intervals comparable to the amounts of enzyme used.

The values for k in the expression $k = \frac{1}{t} \log_{10} \frac{a}{a-x}$ are included in Tables II to IV. A fairly constant value for k is obtained when the progress of the digestion has been considerably slowed by reduction of temperature and concentration of enzyme. As temperature and concentration of enzyme are increased, k be-

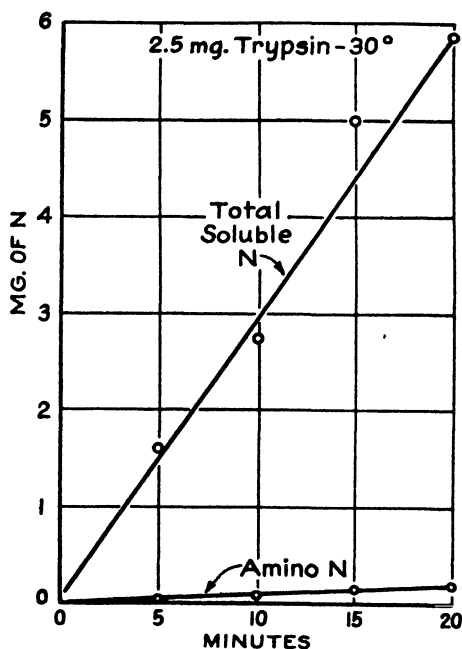


FIG. 5. Showing initial increase of amino nitrogen.

comes increasingly less constant and the time curve becomes more nearly linear.

It was observed that such low values were obtained for amino nitrogen in digestions of very short duration as to raise the question of whether amino nitrogen was produced at all at the very beginning of the digestion, particularly since the total soluble nitrogen had increased noticeably. These results led to a study of the total soluble nitrogen as compared to the amino nitrogen

produced in the very early stages of the digestion. 5, 10, 15, and 20 minute digestions were carried out at 30° with 2.5 mg. of trypsin per digestion. Several blank determinations were made simultaneously. The procedure was similar to that previously described except that all of the filtrate was used for the amino nitrogen determination by the formol titration. The increase in the nitrogen of the total soluble products of digestion was followed by determining the decrease in the total nitrogen of the precipitated casein. Since the difference between the amino nitrogen and the Kjeldahl nitrogen of the blank determinations and that of the actual digestions is only a small portion of the total amount determined, the possibility of error is relatively large, especially in the 5 and 10 minute digestions. The figures, with the probable error in each case, showed that the difference between the average of the blanks and of the 5 minute digestions in the case of amino nitrogen was about 4 times its probable error and therefore that the difference was a true evidence of amino nitrogen production.

Plotting the results as shown in Fig. 5 indicates that the production of amino nitrogen, as compared to the total soluble nitrogen, at the beginning of the digestion is relatively meager.

The temperature coefficient per 10° rise has been stated to be at least 2 for hydrolytic reactions catalyzed by enzymes (8). Cook (9) found that for amylases "at temperatures below the point where the destruction of the enzyme plays an important rôle the rate of hydrolysis is about doubled for every 10° rise in temperature." A comparison of the time intervals required to cause the total disappearance of the original casein or to produce 12 to 15 mg. of amino nitrogen indicates that the temperature coefficient for trypsin in the hydrolysis of casein is about 2.

SUMMARY.

A systematic study of the tryptic hydrolysis of casein has been made by following the progress of production of amino nitrogen, proteose nitrogen, and of the transformation of casein nitrogen into the products of digestion at 20°, 30°, and 40° for several concentrations of enzyme. The data presented show that for all concentrations and temperatures studied the proteose nitrogen reaches a maximum at about the time that the transformation of the original substrate is complete. For all concentrations of enzyme at all

three temperatures a fairly definite relationship holds between the forms of nitrogen studied in that the production of maximum proteose nitrogen, the complete transformation of the original substrate, and the production of a definite quantity of amino nitrogen all occur at about the same time.

A comparison of the time intervals required to cause the transformation of various amounts of casein into the products of digestion is a useful gage of tryptic activity but a similar comparison of proteose nitrogen gives only rough approximations.

In using the production of amino nitrogen as a measure of proteolysis it is essential to choose a comparatively narrow range of amino nitrogen production (about 10 to 12 per cent of the total nitrogen of the substrate) to obtain a relationship of time intervals comparable to the amounts of enzyme used.

A comparison of the time intervals within the range of amino nitrogen production most favorable to a comparison of enzymic activity shows the increase in the rate of hydrolysis per 10° rise in temperature between 20° and 40° to be about 2.

The Van Slyke and Sørensen methods for the estimation of amino nitrogen give practically parallel results in the type of digestion mixture studied.

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VITAMIN D AND FECAL REACTION.

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In previous publications (1), we have described a method for the assay of vitamin D; this method has been criticized by Shohl and Bing (2) and discussed by Oser (3). Shohl and Bing further call attention to a comment on the method recently made by Coward (4).

It seems necessary, therefore, to define what are, in our opinion, the experimental conditions essential if the method is to be used for quantitative assay of the antirachitic vitamin. In none of the experiments described by Shohl and Bing or by Oser have all these conditions been observed, in spite of the fact that they were clearly laid down by us. Moreover, when Shohl and Bing state that we "have suggested that this alteration in pH be used as a test for the cure of rickets," they attribute to us an opinion which we have never expressed and do not hold. What we actually suggested was that the test might be used as a means of measuring vitamin D, not by any means the same thing. For this reason also, the work of Redman (5) on the fecal reaction of rachitic children, though of considerable clinical interest, has no direct relevance to the test described by us.

The "preliminary period" recommended in our first communication was 10 to 14 days, on a defined diet, with a test period of 5 to 7 days. It is not possible in so short a preliminary period invariably to produce characteristic experimental rickets with that diet and still less invariably to cure in the shorter test period any of the histological or chemical changes which are usually agreed to be diagnostic of a rachitic condition.

We found that, with rats of a certain stock, a diet which we described produced characteristic changes in fecal reaction, and that these changes could be reversed when vitamin D was ad-

ministered to the animals, the extent of the reversal being reasonably proportional to the amount of vitamin within certain clearly defined experimental limits. We made no claim to have found any kind of permanent change from alkaline to acid reaction.

To alter the conditions defined by us, and then to claim that the test is not quantitative, or even not specific, is as if to criticize an analytical method for which the reagents and conditions of reaction have been given, on the grounds that it fails if these reagents or conditions are altered. Certainly such investigations, which we are far from decrying, may afford much valuable information as to the *modus operandi* of vitamin D. We have ourselves refrained from expressing opinions on the physiological significance of the phenomena under discussion, since we do not believe the experimental data yet afford a sufficient basis for any definite views.

Oser used diets materially different from our own. Ration 2965 of Steenbock and Black (6), though admirable for producing in rat bones, provided the experimental period is long enough, the characteristic histological picture of rickets and therefore well suited for use in the line test, will not bring about in the period or under the conditions of our test fecal changes sufficiently definite to allow of even qualitative accuracy in detecting vitamin D. Our own experience in this matter confirms that of Oser. Our Diet 401 was described by us in our earlier paper (1).

The reason for the difference in effect of the two diets is almost certainly associated with their different calcium to phosphorus ratios, and possibly also with the actual percentage contents of these elements.

	Diet 2965.	Diet 401.
Ca.....	1.18	0.42
P.....	0.33	0.07
Ca:P.....	3.6	7.0

Shohl and Bing write, "The slight alteration in pH with Steenbock's diet as compared with the greater shift with Zucker's diet may be associated with their different potential alkalinities."

No reference was given by these authors as to their method of determining "potential alkalinities," and we unfortunately only found the reference in a paper by Shohl, Bennett, and Weed (7) to the method of Sherman and Gettler (8) too late to incorpo-

rate results based upon this method in this paper. It must, therefore, for the moment remain an open question whether the different behavior of the two diets is attributable to their different "potential alkalinities" or to their different phosphorus to calcium ratios, or to both.

We are fully aware of the temporary nature of the change in fecal pH, and have ourselves observed the rhythmicity to which Oser calls attention. We also agree with him that the buffering of the feces renders unimportant the exact degree of dilution of their aqueous suspension, within fairly wide limits. We have never found any need to take the special precautions described by Shohl and Bing for collecting and extracting the feces, and we have not been able effectively to replace the rocking hydrogen electrode by the quinhydrone electrode, possibly owing to the presence of variable amounts of disturbing oxidation-reduction systems in the feces.

pH determinations, with both the hydrogen and the quinhydrone electrodes, have been made here by Mr. E. L. Smith on 50 different aqueous suspensions of rat feces. The range of pH determinations with the hydrogen electrode was from 5.69 to 7.73, and with the quinhydrone electrode from 5.63 to 8.08. In twenty-two cases the hydrogen electrode gave higher readings and in twenty-eight cases lower. The extreme variations were shown when the quinhydrone electrode showed 8.08 and the hydrogen electrode 7.66, and when the hydrogen electrode showed 7.45 and the quinhydrone electrode 7.10. The mean difference, signs being ignored, was 0.16, and when signs were allowed for, 0.03; the differences were, that is to say, random differences.

It is essential that only perfectly healthy animals, whose feces show the required degree of alkalinity in a relatively short time, be used if the test is to have quantitative validity. Recently a certain amount of difficulty has been experienced here in using animals which, possibly owing to a very low vitamin A reserve, have shown a marked tendency to gastrointestinal trouble, with occasional diarrhea and even hemorrhage. Such animals on Diet 401 may maintain only a slightly alkaline or even a neutral fecal reaction for 15 or 20 days, and are useless for the test.

Further, the test is only quantitatively successful if relatively large doses of vitamin D can be administered in relatively small

quantities of vehicle. Substances such as butter, irradiated food products (especially those containing large quantities of fat or sugar) have been found to induce effects in fecal reaction quite disproportionate to their low but known content of vitamin D—an effect analogous to that found by Bergeim (9) with lactose; it is to be noted that all these substances would have had to be fed in quantities of 2 gm. or more per day if the animals were to receive vitamin D adequate to produce the fall in fecal pH which we have chosen as our standard. This standard was itself based on the effect found to be produced with 100 mg. of the best of a very large number of samples of high grade cod liver oil.

The failure to realize that this test can only work quantitatively with relatively large doses of vitamin D in relatively high concentration has doubtless led to the comments made by Shohl and Bing on two of the curves shown in our first paper, representing fecal pH changes in animals submitted to direct irradiation. Even had they used a suitable basal diet, they would probably still have found very small, and not significant, changes in fecal pH when their animals were changed from a non-irradiated to an irradiated basal diet.

The extent to which any foodstuff, or animal, can be irradiated depends on several variables, among which the most fundamental is the actual content of provitamin D. With ordinary dietary constituents this is probably so small that irradiation under optimum conditions (usually not achieved) may produce just sufficient vitamin D to protect an animal fed exclusively or largely on the irradiated material, but far too little to cause any definite positive response when the material is fed as a supplementary substance in the fecal pH test, where at most 2 gm. per day can conveniently be administered with any certainty of complete consumption.

The case with direct irradiation of the experimental animals is very similar. The curves relevant to the matter shown in our first communication were inserted solely to demonstrate the qualitatively similar effects of irradiation and oral vitamin D administration. We were, in fact, rather surprised to obtain so definite a fall in pH as that shown; this was transient in precisely the same manner as that due to direct vitamin D administration, and was suggestively greater in the animals submitted to the longer period of irradiation. But we had no intention of advocating the albino rat as a practical form of ultra-violet photometer.

If materials which are definitely antirachitic, but which are in no sense concentrated sources of vitamin D, are to be assayed by the fecal pH test, one of two alternative procedures may be adopted. After extraction of the material with ether, either the ether-soluble matter or its unsaponifiable fraction can be dissolved at a

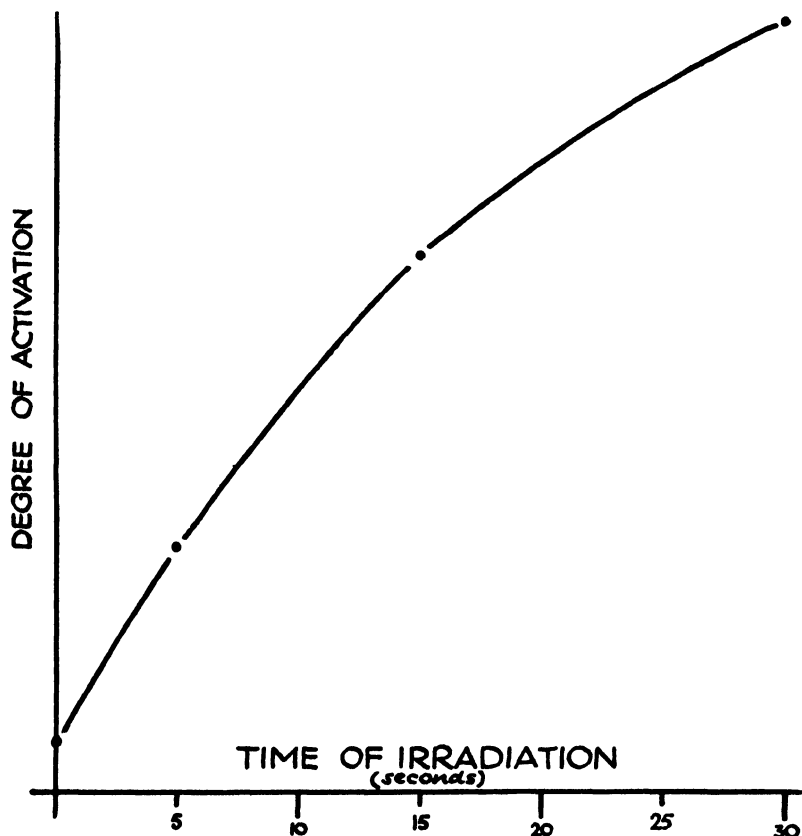


FIG. 1. Effect of time of ultra-violet irradiation on vitamin D content of cereal food.

degree of concentration sufficiently high and can then be fed directly as a supplementary ration to test animals. The second procedure is based on a suggestion of Steenbock (private communication) to replace part of the most comparable constituent in the basal diet by an equivalent amount of the material under test.

This is more expeditious than the first procedure outlined above, but we made use of it with some misgiving, since the weighing back of food, and the integration of the amount of test material taken over the whole period, seemed to us to involve a counterbalancing complication and to be a probable source of considerable inaccuracy.

We were, therefore, very pleasantly surprised to find that a preliminary experiment indicated considerable uses for this modified method. By the courtesy of the Quaker Oats Company, of Chicago, we were put in possession of four samples of a cereal three of which had been irradiated for different times under standard conditions. These cereals are referred to below as follows: Cereal Q 1 unirradiated, Cereal Q 2 irradiated 5 seconds, Cereal Q 3 irradiated 15 seconds, Cereal Q 4 irradiated 30 seconds. These were incorporated in basal Diet 401 to the extent of 25 per cent of the whole, replacing approximately 30 per cent of the patent flour. Four pairs of animals were fed Diet 401 with Cereal Q 1 incorporated. In the case of three of the pairs of animals, when their fecal pH had reached the necessary value, the diet was changed to diets incorporating Cereals Q 2, Q 3, and Q 4 respectively. The resulting values of π (the minimum mean fecal pH as defined in our first communication) were as follows:

Cereal	π
Q1	7.16
Q2	6.97
Q3	6.76
Q4	6.57

If it be assumed that, on the average, the daily consumption by the animals of diet, and therefore of test material, was identical, it is possible to plot the relative vitamin D contents against the time of irradiation of the test material (Fig. 1).

A second test, carried out on lines similar to the above, gave similar, though not so strikingly regular, results.

We have mentioned above a criticism of the pH test, made originally by Coward, and referred to by Shohl and Bing. Coward's actual words are, "The 'line' test is to be preferred to such a method as Zucker's (pH of the fæces) in that it is a direct measure of the change which the vitamin is intended to produce clinically."

We would make two comments on this view. It is, in our opinion, more or less of an experimental accident that rickets is used as a test disease for vitamin D. The mode of action of this vitamin may be little, if any, better understood than that of the other five, but it is certainly not confined exclusively to the prevention or cure of rickets, for otherwise adults could thrive in its absence. An array of clinical data can be mustered to bear witness to its importance in restoring or maintaining normal calcium-phosphorus metabolism, and therefore for general health. Provided a reasonably specific test for vitamin D can be established, there is no object in insisting that its ability to prevent or cure rickets should be made the basis of the test. In other words, we do not admit Coward's contention that a laboratory test should necessarily always remain an attempt to reproduce clinical conditions, nor do we consider that vitamin D must be valued solely, or even mainly, by its effect on rickets, clinical or experimental.

The specificity of the pH test depends on a knowledge of the conditions under which it will work, and on the exercise of the reasonable minimum of care and skill required to maintain these conditions. In our earlier communications we have described positive results obtained for the most part with cod liver oil or its unsaponifiable fraction and negative results obtained with material definitely known or generally held to be inactive. A considerable amount of further experience of this method, involving the use of several hundreds of animals as a means of assaying irradiated ergosterol, has not caused us to modify in any way our view of its usefulness for rapidly assaying highly concentrated preparations of vitamin D.

SUMMARY.

The assay of vitamin D, based upon examination of the fecal pH of rats on a rachitogenic diet, as described by us elsewhere, has been found to have a continued useful application, provided the experimental conditions laid down by us are rigorously observed with reference to the diet, the size and concentration of the dosage, the condition of the animals, and the period of the test, etc. Some recent publications which criticize or comment on the test are discussed from this point of view.

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THE RESPIRATORY PROTEINS OF THE BLOOD.

IV. THE BUFFER ACTION OF HEMOCYANIN IN THE BLOOD OF LIMULUS POLYPHEMUS.

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Although the buffer action of the blood of man has received most thoroughgoing analysis (Van Slyke, 1921) the similar phenomena in the body fluids of invertebrates have been described only in such a way as to define the magnitude of the problem without at all adequately contributing to its solution. Quagliariello (1916) showed that relatively large amounts of acid or alkali must be added to the blood of certain invertebrates in order to produce a given change in its reaction. Thus in the cephalopods, *Eledone* and *Octopus*, some 200 mg. of sodium hydroxide were required to bring the reaction of a liter of blood from its normal value of pH 7.36 or 7.54 to pH 9.0. In the case of the horseshoe crab, *Limulus polyphemus*, we find that 400 mg. of sodium hydroxide are required to produce this effect. This is 1000 times the amount required to produce a comparable effect in distilled water. To what components of the blood is this large buffer effect due?

Parsons and Parsons (1923-24) amplified the observations of Quagliariello by working out the carbon dioxide dissociation curves of a number of marine invertebrates. They point out that those bloods which combine with carbon dioxide in quantity are rich in hemocyanin. On the other hand Nitzescu and Cosma (1927) conclude from a study of the carbon dioxide dissociation curve of the snail, *Helix pomatia*, that in this species protein substances (hemocyanin) do not play the principal rôle in the acid-base equilibrium. Redfield, Coolidge, and Hurd (1926)

point out that the carbon dioxide-combining power of different species is not strictly comparable to their hemocyanin content as judged by the capacity for uniting with oxygen. This may be due entirely to specific differences in the properties of hemocyanins of different organisms or it may be attributable to varying quantities of other buffer substances in these bloods. We have attempted to evaluate these alternatives in the case of the blood of *Limulus polyphemus*. This blood is well adapted to the inquiry because it is one of those in which the buffering action is largest—relative to oxygen capacity—and in which one might expect to find other buffer substances taking an important part.

The substances known to be of significance as buffers in mammalian blood are the carbonates, phosphates, and proteins. The very interesting and important buffering action, described by Collip (1920–21, 1921) due to calcium carbonate of the shells of certain pelecypods and crustacea need not concern us here, since we have defined our problem in terms of phenomena observable in the blood *in vitro*. *Limulus* blood may contain as much as 10 or 20 volumes per cent of carbon dioxide, equivalent to 0.005 or 0.010 mols of bicarbonate per liter. While this bicarbonate is conceivably of importance in buffering other acids, because of the ease with which the highly diffusible carbonic acid may be eliminated through the gills, from another point of view the bicarbonate exists in the blood because carbonic acid has been buffered, rather than as one of the primary buffer substances of the blood. This is shown by the fact that when the carbon dioxide pressure is reduced to zero the blood no longer contains more than a trace of carbonate, the base normally bound as bicarbonate having reverted entirely to the other buffers of the system.

The importance of phosphates as buffers in *Limulus* blood may be approximated from analyses of the ash which have been made by Genth (1852), Gotch and Laws (1884), and McGuigan (1907). McGuigan, whose values are typical, found the blood to contain 2.676 per cent ash, of which 0.34 per cent was estimated to be P_2O_5 . If all this phosphorus were present in the blood as salts of phosphoric acid, their concentration would be about 1.3×10^{-3} molar. In changing from a reaction of pH 7.3 to 9.0 less than half of the phosphate would be converted from the primary to the secondary phosphate. Consequently

the phosphate could not combine with more than about 0.5×10^{-3} mols of base per liter. We have found, however, that *Limulus* serum will combine with about 400 mg. of NaOH, or 10×10^{-3} mols per liter, in undergoing this change in reaction. The phosphates, therefore, cannot account for more than 5 per cent of the total buffer action in the blood of *Limulus*. That inorganic constituents should not be of importance in this regard is not surprising when it is considered how little this animal regulates the salt content of its serum (Macallum, 1910).

The blood of different individuals of *Limulus* differs greatly in its oxygen- and carbon dioxide-combining power, depending on how long and under what conditions the animal has been kept in captivity. This has enabled us to compare the buffering power of bloods naturally differing in protein content. Using the usual technique (Redfield, Coolidge, and Hurd, 1926) we have determined the carbon dioxide bound by four specimens of serum at different carbon dioxide pressures. These data are illustrated by the points in Fig. 1. Through these points have been drawn curves spaced relative to one another as they should be if the carbon dioxide-combining power is proportional to the nitrogen content of the blood. This has been done in accordance with the principle that at any hydrogen ion concentration the quantity of carbon dioxide combined will be proportional to the quantity of buffer present. Consequently we have drawn a standard curve through the points yielded by the serum containing 8.95 mg. of nitrogen per cc. Through this we have drawn a number of lines which converge at the origin. These lines are each the locus of a single hydrogen ion concentration. Each theoretical curve was then constructed so as to transect each of these lines of equal hydrogen ion concentration at ordinates bearing the same proportions to the ordinate of the standard curve, as the nitrogen content of the specimen bears to the nitrogen content of the standard curve. It may be seen from Fig. 1 that theoretical curves drawn in this way very adequately describe the position of experimental points obtained in bloods having a 4-fold variation in nitrogen content. This result indicates that the buffering of *Limulus* blood must be attributed largely to its nitrogenous compounds. What nitrogenous substances does it contain?

Non-protein nitrogenous substances occur in almost negligible quantities in this blood, there being only 0.12 gm. of nitrogen non-coagulable by heat in 1000 cc. of serum according to our analyses. Alsberg (1914) observed as much as 0.45 gm. per 1000 cc. in certain specimens.

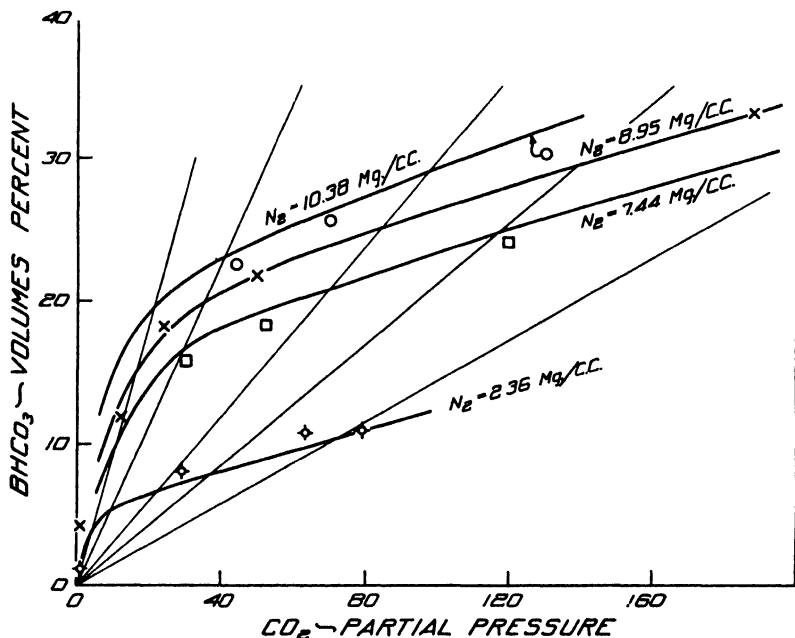


FIG. 1. Carbon dioxide dissociation curves of *Limulus* serum of different nitrogen content. Abscissæ represent pressure of carbon dioxide in mm.; ordinates, combined carbon dioxide (BHCO_3) in volumes per cent. The curves are drawn through the experimental points on the assumption that the combined carbon dioxide (BHCO_3) is proportional to the nitrogen content of the specimen.

Alsberg (1914) inquired into the protein content of *Limulus* serum and concluded that in addition to hemocyanin there was another protein present derived from the blood corpuscles in the process of clotting. In quantity he considered it insignificant compared to the total protein content. Redfield, Coolidge, and Shotts (1928) point out that purified hemocyanin contains more copper per gm. of protein nitrogen than does serum. The

difference is such (90×10^{-4} mg. of Cu per mg. of N in the case of serum as compared with 99×10^{-4} mg. of Cu per mg. of N in the case of hemocyanin) as to indicate that not more than 10 per cent of the protein is other than hemocyanin. Whether this other protein is present in the native blood one cannot say: undoubtedly it was present in the sera with which we have experimented.

The foregoing considerations suggest strongly that hemocyanin is the principal buffer substance of *Limulus* blood. If so, it should be possible to imitate the buffer action of blood by similar solutions of purified hemocyanin. We have consequently prepared titration curves of *Limulus* serum, and of purified hemocyanin dissolved in water and in solutions of NaCl and mixtures of NaCl and $MgCl_2$. From such titration curves the buffer value at any hydrogen potential may be deduced and used as a basis for comparing the properties of the different solutions.

Titration Curve of Limulus Serum.—This was obtained from the blood of a single animal freed of clot by shaking and filtering. The specimen was preserved with toluene and kept on ice during the several days required for the measurements. Measurements of hydrogen potential were made with the hydrogen electrode upon samples prepared by adding 10 cc. of water containing varying quantities of HCl and NaOH to 10 cc. of serum. These samples were allowed to stand overnight in the cold and were then placed in a tonometer which was evacuated in order to free the samples of oxygen and carbon dioxide. Hydrogen was then introduced into the tonometer and the sample saturated with this gas by rotating the tonometer for 10 minutes. The sample was next passed into the Clark electrode vessel which was rocked for 30 minutes before the potential was measured. Hydrogen potentials were estimated, the potential of the 0.1 N calomel electrode being taken as 0.3356 at 25°.

The quantity of hemocyanin present in the stock specimen of serum was estimated from its copper content. Analyses of three samples by the method described by Redfield, Coolidge, and Shotts (1928) yielded 0.0878, 0.0884, and 0.0888 mg. of Cu per cc. of solution. Taking copper to compose 0.173 per cent of *Limulus* hemocyanin, we estimate that the serum contained 0.0511 gm. of hemocyanin per cc. The samples on

which measurement was made were diluted with an equal volume of water containing acid or alkali and consequently contained approximately 2.5 per cent hemocyanin.

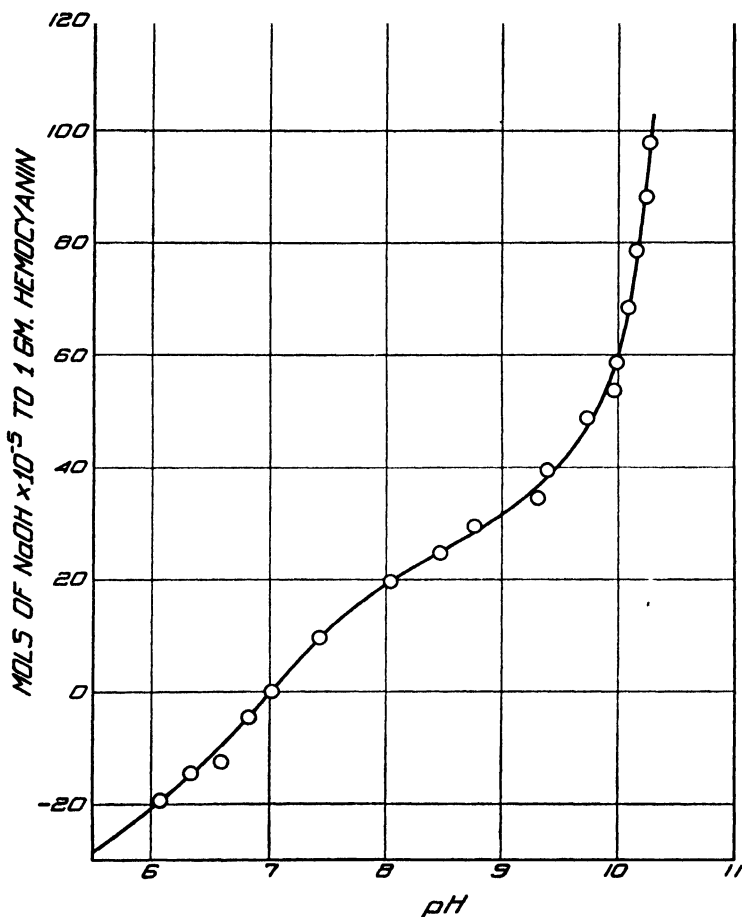


FIG. 2. Titration curve of *Limulus* serum. The quantity of NaOH added per 10^5 gm. of hemocyanin present in the serum is indicated along the ordinate. Negative values on the ordinate indicate the addition of equivalent quantities of HCl to the serum.

The titration curve of reduced *Limulus* serum so obtained is shown in Fig. 2. The values of hydrogen potential plotted are the best representative values of a series of two to six different

measurements of the same sample. The temperature at which the measurements were made varied between 23–25°.

Titration Curves of Solutions of Hemocyanin.—These were obtained from specimens made from Preparation XIIA of Redfield, Coolidge, and Shotts (1928). This preparation had been purified by repeated salting out with ammonium sulfate, followed by dialysis until free of sulfate. It contained 0.10 gm. of hemocyanin per cc. as estimated from the dry weight; of this weight 0.167 per cent was copper. By diluting 2 cc. of hemocyanin with 8 cc. of water, containing various amounts of HCl or NaOH, specimens were obtained containing 2 per cent hemocyanin and of varying hydrogen potential. These samples were saturated with hydrogen and their hydrogen potential measured in exactly the same way as were the samples of serum. The temperature of the electrode vessels was 21–23°.

The preparation employed contained 26×10^{-5} mols of NaOH per gm. of hemocyanin as determined by the quantity of dilute HCl required to produce the maximum precipitation of the hemocyanin in the region of the isoelectric point. In order to determine the quantity of NaOH present in the samples measured this quantity was added to the amount of NaOH mixed with the sample in preparing the final dilution. When HCl was added to the sample the quantity added per gm. of hemocyanin was subtracted from 26×10^{-5} in order to give the amount of NaOH left unneutralized in these preparations. The quantity of NaOH present per gm. of protein thus obtained is plotted as the ordinates of titration Curve A in Fig. 3.

The measurements have not been corrected for the amount of base remaining uncombined because this is not a significantly large quantity below pH 11. Beyond this reaction the hemocyanin undergoes some change, as indicated by the appearance of a purple color similar to the biuret reaction, which would appear to invalidate any conclusions which might be drawn concerning the maximum base-binding power of hemocyanin.

The titration curve of pure hemocyanin is clearly quite different from that of serum. Whereas the former rises in a rather uniform slope from the point of neutrality to pH 10.5, after which it is inflected upward with great rapidity, the latter rises more steeply between pH 6.5 and 7.5; it is then flattened out

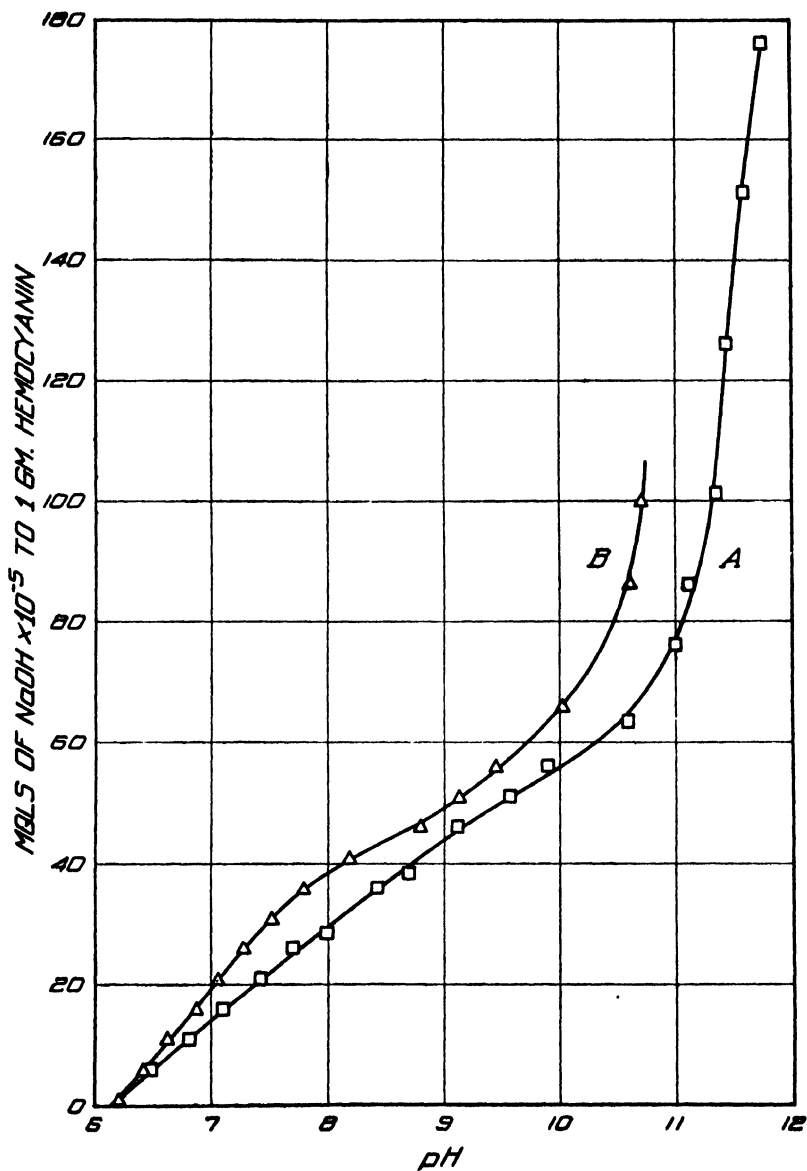


FIG. 3. Titration curve of *Limulus* hemocyanin; Curve A in the absence of salt; Curve B in the presence of 0.5 M NaCl.

between pH 8.0 and 9.0, after which it again rises steeply in the region about pH 10.0.

Titration Curve of Hemocyanin in the Presence of Neutral Salts.—From the fact that the addition of salt greatly alters the equilibrium between hemocyanin and oxygen, and that it also modifies the color of this substance, it was natural to suspect that the addition of neutral salts to the hemocyanin solution would modify the titration curve also. Sørensen, Linderstrøm-Lang, and Lund (1927) have recently given a theoretical interpretation of such phenomena as observed by them in solutions

TABLE I.
Composition and Ionic Strength of Limulus Serum.

	<i>Limulus</i> serum	Ionic concentration	Concentration × valence ²
	<i>gm per l</i>		
Na	8 885	0 386	0 386
K	0 4589	0 0127	0 0127
Ca	0 3613	0 00903	0 0361
Mg	0 9955	0 0410	0 1640
Cl	16 608	0 468	0 468
SO ₄	1 1185	0 0116	0 0464
Hemocyanin	50		
Total			1 1132
Ionic strength			0 5566

of egg albumin, and Simms (1929) has studied similar effects in gelatin preparations.

The titration Curve B in Fig. 3 was obtained from a 2 per cent solution of hemocyanin containing NaCl in the concentration of 0.5 mol per liter. The hemocyanin was the same preparation used in the previous series of measurements, and the measurements for the two curves were actually made in alternation.

The addition of NaCl brought the titration curve of purified hemocyanin much closer in shape to that of the native serum. At hydrogen potentials below pH 9.0 the curves are much alike. The abrupt rise on the alkaline end of the curve occurs sooner than in the salt-free hemocyanin but not as soon as in

the serum. The reason for the steep rise in the titration curve of serum is obvious when it is recalled that *Limulus* serum

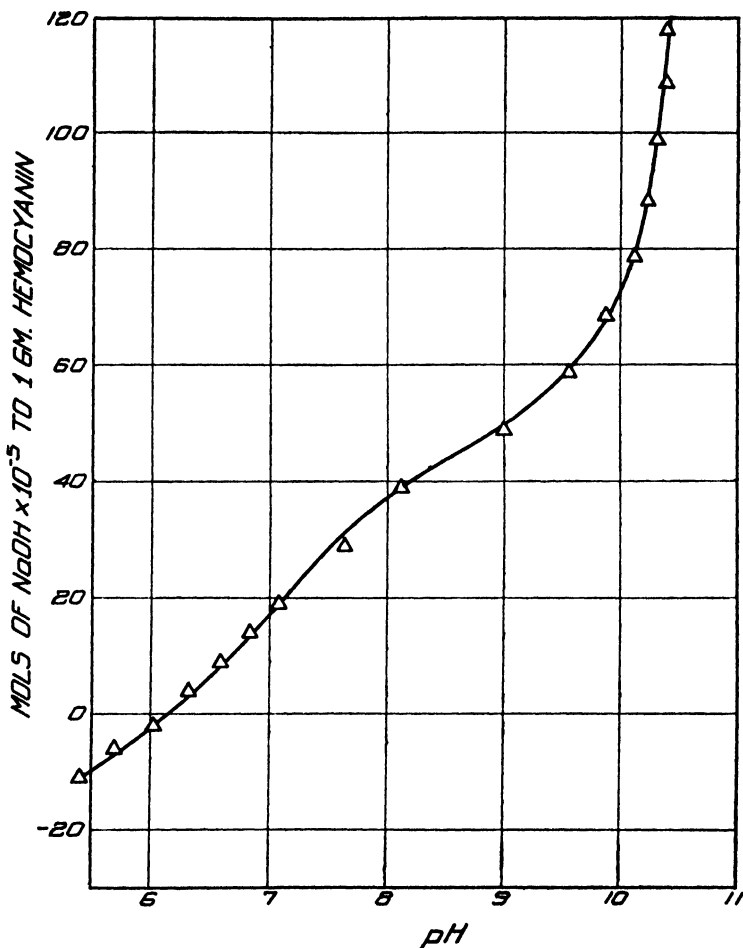


FIG. 4. Titration curve of *Limulus* hemocyanin in the presence of NaCl and $MgCl_2$. The quantity of NaOH present in excess of HCl is indicated along the ordinate. Negative values indicate an excess of HCl over NaOH.

contains a considerable quantity of magnesium, the hydroxide of which becomes insoluble at about pH 10. The titration curve of sea water, which contains much the same quantity of

magnesium as does *Limulus* serum, has a similar steep upward inflection just beyond pH 10 (Haas, 1916).

We have attempted to imitate the titration curve of *Limulus* serum with a solution in which the concentration of hemocyanin and magnesium is the same as that in serum and having a total ionic strength equal to that of serum secured by the addition of sodium chloride. Table I gives the composition of *Limulus* serum based on Macallum's (1910) analyses for inorganic salts and our estimate of the hemocyanin content of the specimen of which we have determined the titration curve. The ionic strength of the known components, with the exclusion of the hemocyanin is 0.5566.

We prepared a mixture containing 0.44 mols of NaCl, 0.04 mols of $MgCl_2$, and 50 gm. of hemocyanin per liter. This solu-

TABLE II.
Excess of Base Present in Natural Limulus Serum.

Sample No.	pH	Excess of base. Mols per 10 ⁶ gm. hemocyanin.
14	7.2	22
16	7.4	28
29	7.9	36
33	7.0	18
35	8.0	37

tion has an ionic strength of 0.56 and a hemocyanin and magnesium content very similar to native serum. Samples of this solution were diluted with equal volumes of water containing NaOH or HCl and the hydrogen potential measured. The temperature at which the measurements were made varied between 21–23°. These data corrected for the combined base in the hemocyanin, are presented in Fig. 4.

Comparison of the titration curves of serum and of this mixture of NaCl, $MgCl_2$, and hemocyanin shows that the curves are not only very similar in shape but that they have identical slopes at all reactions between pH 6 and 10. Beyond this point there is a slight discrepancy due to the failure of the magnesium in the artificial mixture to duplicate entirely the conditions existing in serum.

Since the slope of the titration curves of the artificial mixture and the natural serum is the same at all physiological reactions, one may conclude that within the limits of experimental error the buffering of *Limulus* serum within this range may be accounted for by its hemocyanin.

Aside from the discrepancy at extreme alkalinity the difference in the curves in Figs. 2 and 4 consists solely in the fact that the ordinates of the curve for the artificial mixture are 18×10^{-5} mols per gm. greater than those of the natural serum at corresponding hydrogen potentials. This difference clearly represents the quantity of base, in excess of acid, present in the natural blood. Consequently Fig. 4 may be used to determine approximately the quantity of base present in any sample of *Limulus* blood of which the hydrogen potential is known. Such determinations on a number of samples are given in Table II.

While it may be concluded that hemocyanin is the only significant buffer in *Limulus* serum, it is equally clear that its value in this regard is dependent to an important degree upon the electrolytic environment which the salts of serum provide. The influence of the other components of the solution upon the buffering of hemocyanin is clearly brought out by estimating the molecular buffer values of the solutions at various hydrogen potentials. The molecular buffer value is defined by Van Slyke (1922) as

$$\beta_M = \frac{dB}{Cd pH}$$

where B is the molecular concentration of base added and C is the molecular concentration of the buffer substance. Since we are dealing with measurements made on reduced solutions of hemocyanin, we designate their molecular value by the symbol β_a . The molecular concentration of hemocyanin is estimated on the assumption that its molecular weight is 73,400 (Redfield, Coolidge, and Shotts, 1928). While this value is not securely established, it represents the weight of *Limulus* hemocyanin containing 2 atoms of copper and which Redfield, Coolidge, and Montgomery (1928) have shown to be equivalent to 1 molecule of oxygen. In consequence our values of β_a are strictly compar-

able with those of Van Slyke and his collaborators (1922) who use the equivalent oxygen-combining capacity of hemoglobin in

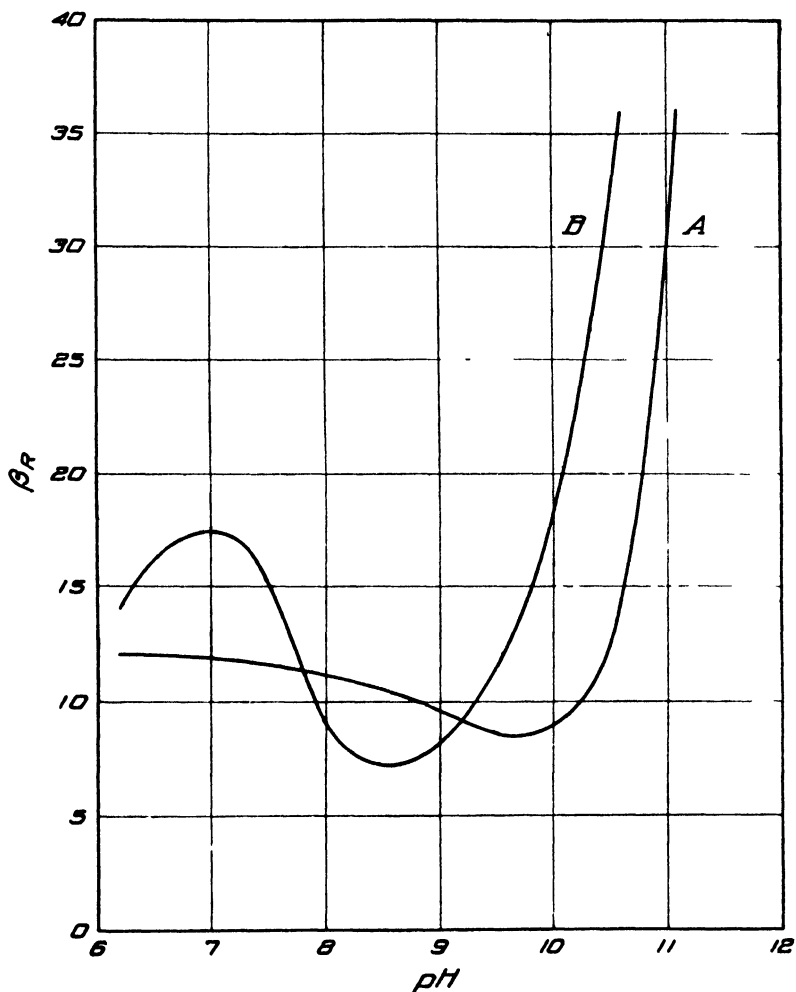


FIG. 5. Molecular buffer values, β_R , of *Limulus* hemocyanin solutions: Curve A in the absence of salt; Curve B in the presence of 0.5 M NaCl.

estimating the buffer values of this substance. The value of β_R has been estimated by multiplying the "slopes" of the titration curves in Fig. 3, which give buffer values in mols of base

per 10^5 gm. of hemocyanin, by 73,400 to obtain the molecular buffer value as defined. The results of this estimation are shown in Fig. 5 which brings out the following relations.

1. The molecular buffer value of reduced *Limulus* hemocyanin in the absence of salts varies very little over a range of pH from 6.0 to 10.

2. The presence of 0.5 mol of NaCl, presumably through altering the dissociation constants of certain of the acidic groups of the hemocyanin molecule, causes a considerable increase in the buffer value below pH 8 at the expense of the buffer value between pH 8 and 9. As a result the effectiveness of hemocyanin as a buffer in the physiological range is increased up to some 40 per cent.

3. Compared on the basis of equivalent oxygen capacity the buffer value of reduced hemocyanin is about 4 times as great as that of hemoglobin. This difference is to be attributed to the inferiority of hemocyanin as an oxygen carrier, rather than to any superiority of this protein as a binder of base, for when the buffer value is estimated in terms of base bound per gm. of protein: β_r for hemocyanin is about 15 mols per gm. of protein per pH unit and β_r for hemoglobin is 15.9. Weight for weight there is little difference in the buffer values of these two proteins.

Change in Buffering Due to Oxygenation.—The foregoing estimates of buffering power in *Limulus* blood have been made on the basis of experimental measurements on solutions containing hemocyanin in the reduced condition. It is well recognized that in the blood of mammals a very considerable part of the effective buffering of the blood is due to changes in the strength of its acidic groups brought about simultaneously with the reduction of the hemoglobin. Kerridge (1926) has shown that a similar phenomenon occurs in the blood of the crab, *Maia squinado*, and Redfield, Coolidge, and Hurd (1926) have demonstrated the same relation in the blood of the squid, *Loligo pealei*. The latter authors were unable to demonstrate any difference in the carbon dioxide content of oxygenated and reduced blood of *Limulus polyphemus* under comparable carbon dioxide pressures. Consequently the influence of oxygenation upon the buffering of *Limulus* blood must be very small.

SUMMARY.

1. Phosphates and proteins other than hemocyanin are present in quantity sufficient to account for only a small part of the buffering of *Limulus* blood.

2. The titration curve of pure hemocyanin does not closely reproduce that of serum.

3. The addition of NaCl to hemocyanin alters the titration curve so that it approaches more closely to that of serum.

4. A solution containing hemocyanin and magnesium chloride in amounts comparable to serum, and having the total ionic strength of serum made up by the addition of NaCl, has a titration curve very similar to that of serum.

5. It is concluded that the properties of hemocyanin as influenced by its electrolytic environment will account for the buffer phenomena of *Limulus* blood.

6. Samples of natural *Limulus* serum are found to contain from 18 to 37×10^{-5} mols of base, in excess of acid per gm. of hemocyanin.

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THE INFLUENCE OF NEUTRAL SALTS ON THE pH OF PHOSPHATE BUFFER MIXTURES.

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(Received for publication, April 5, 1929.)

The results embodied in this report were obtained as preliminary data for a systematic study of the magnitude of errors produced by the presence of various substances in the use of phenol red for colorimetric determinations of hydrogen ion concentration in biological fluids. Neutral salts, present in all body fluids, are known to have an influence on dye color. There are available many tables of salt errors of indicators. In most cases these are given for one buffer mixture and one indicator concentration. The question arises as to the constancy of the salt error with change in indicator concentration, with change in the buffer concentration, and with change in the pH. Before this can be definitely answered it is necessary to study the effect of neutral salts on the true hydrogen ion concentration (as measured by the hydrogen electrode) for the entire range of the buffer and at various dilutions. Sørensen and Palitzsch (1) drew attention to the fact that the addition of neutral salt to buffer solutions altered the electrometric pH and pointed out that the entire change as observed by a colorimetric reading could not be called a salt error of the indicator.

It is a well recognized fact that the addition of small amounts of neutral salt to dilute buffer solutions increases the hydrogen ion concentration as determined by the potential of the hydrogen electrode. At the present time all other electrometric and also colorimetric, titrimetric, and gasometric methods for the determination of H^+ are standardized directly or indirectly by a hydrogen electrode system. In order to note the effects produced by a

* Presented to the Graduate School of Vanderbilt University in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

substance on these simpler and under most conditions more convenient methods, it is necessary that their effects as measured by the hydrogen electrode be carefully considered.

It was decided to study first the effects of addition of neutral salt to the phosphate buffer system because it is the one used extensively in biological studies, both as a buffering agent and as standards for colorimetric pH determinations with phenol red in the range of pH 7 to 8. Sørensen used the salts $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and KH_2PO_4 in $\text{m}/15$ concentration. Some investigators have experienced trouble with $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in that it became more hydrated. The acid salt crystallizes out of solution easily and the anhydride is stable. Therefore, Clark and Lubs (2) recommended that the phosphate standards be prepared from KH_2PO_4 and standardized NaOH solution. Cullen (3) has used the $\text{m}/15$ phosphate mixtures of Sørensen. When, because of war conditions, it became impossible to import the $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ salt, he asked Merck and Company, Inc. to prepare it. They prepared instead the anhydrous salt which has been entirely satisfactory for many years. This method is more convenient for most biological laboratories as it does not involve the preparation and keeping of pure, CO_2 -free, sodium hydroxide. It has been the experience in this laboratory that the salts as furnished by the above manufacturer are reliable and convenient for the preparation of the solutions. The values obtained for the pH of mixtures made from these salts have checked consistently within experimental errors the values as given by Hastings and Sendroy (4) when the system was standardized in the same manner. Myers and Muntwyler (5) have recently used these salts in the preparation of their standards to be used in the wedges of the Myers colorimeter. As these salts are being generally used, it seemed advisable to continue their use in the present study. Considerable new information has been obtained. An attempt has been made to interpret these results in accordance with the Debye-Hückel concept of the influence of interionic forces (6, 7).

EXPERIMENTAL.

Preparation of Solutions.

Solutions of $\text{m}/7.5$ Na_2HPO_4 , $\text{m}/7.5$ KH_2PO_4 , and 10 per cent solutions (10 gm. of salt dissolved in water and made up to 100 cc.

in a volumetric flask) of NaCl and KCl were prepared from Merck's Blue Label reagents. Stock solutions of the buffer mixtures were made for each series of determinations. For example, when the effect of a concentration of NaCl of 0.5, 1, and 2 per cent in a $m/15$ phosphate mixture containing 8 parts of basic salt and 2 parts of acid salt was determined, a stock solution of a $m/7.5$ phosphate mixture containing that ratio of salts was first prepared. To each of four 100 cc. volumetric flasks, 50 cc. of the phosphate mixtures were added by means of a delivery pipette and to the respective flasks, no NaCl, 5 cc., 10 cc., and 20 cc. of a 10 per cent solution of NaCl. The flasks were then made up to mark with distilled water. In this manner one obtains an exact phosphate ratio in each of the flasks. Approximately the same procedure was carried out in the entire series of determinations indicated in Tables I to III. In all cases the solutions were kept in Pyrex flasks.

Electrometric pH Determination.

The electrometric determinations of hydrogen ion concentration were made by the customary method with

Saturated calomel electrode	Saturated KCl bridge	Electrode liquid	H ₂ -Pt
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The present study was made at 38° for the reason that it was desirable to secure data at 38° to use in studying the corrections necessary in the use of phenol red for colorimetric pH determinations in serum and tissue fluid. Considerable data are available for room temperature while there is but little available for blood temperature. The temperature was controlled within $\pm 0.1^\circ$. The 2.5 cc. Clark-Cullen temperature-controlled electrode vessel was used with the saturated calomel electrode. Potential measurements were made with a Leeds and Northrup "hydrogen ion" potentiometer, enclosed lamp and scale galvanometer, and a Weston standard cell. The hydrogen used was furnished by the International Oxygen Company. The details of the method, preparation and care of electrodes were as described by Cullen (3). The individual pH determinations reported here are accurate to at least 0.005 pH.

At the beginning of a series of determinations 0.1 N HCl, prepared from constant boiling acid obtained by the method of Hulett and Bonner (8), was placed in the electrode vessel and the potential measured. The pH of 0.1 N HCl was given the value of 1.08 and the e of the system was calculated, $\text{pH}_{38^\circ} = \frac{\text{E.M.F. } e}{0.06169}$.

This was the method used for most of the determinations. In a few the value of e was determined with a phosphate mixture that had previously been standardized with 0.1 N HCl. In every case the standardizing solution was again run at the end of the series in order to make sure that no change had taken place in the system. Determinations were made in duplicate and refills were made until the readings of the two cells were constant and in agreement.

Standardization and Use of the Term pH.

0.1 N HCl, with the pH value assigned at 1.08, has been used as the final standard of reference as suggested by Cullen (3). The reasons for using this standard were again discussed by Cullen, Keeler, and Robinson (9). It is now widely used in American biological laboratories. The value of 1.08 at 38° was obtained by the use of the activity values for 0.1 N HCl given by Noyes and Ellis (10). Owing to the lack of information on the change of activity values with temperature the value 1.08 was adopted for all temperatures between 15–40°. In practically all the work that has been done in the past with the use of M/15 phosphate buffers, the pH values given to the various mixtures are those of Sørensen. His values were obtained at 18° by using the Bjerrum extrapolation for liquid junction potential and assigning to hydrochloric acid a value based on conductivity measurements. It has been previously pointed out (Cullen, Keeler, and Robinson (9), Hastings and Sendroy (4)) that the values of the pH of M/15 phosphate buffer mixtures at 20°, determined electrometrically without correction for liquid junction potentials and with the Cullen standardization, check within 0.01 pH unit the pH values given by Sørensen for 18°, and that at 38° the phosphate mixtures give a pH value approximately 0.03 pH more acid. The latter difference has been obtained repeatedly and used by Cullen, Keeler, and Robinson and by Hastings and Sendroy. It seems to be constant for the entire range of the phosphate buffer mixture. In

view of the present lack of knowledge of the absolute value of the hydrogen ion activity in a reproducible standard solution, and since it is hoped that the present data will prove useful in connection with previous work, it seems logical to continue to use the HCl standard and to retain the term pH instead of adopting the term $p_{a_{H^+}}$ suggested by Sørensen and Linderstrøm-Lang (11). At the present time there is a confusion in regard to this latter term. It is important to realize that the pH values given in this paper are identical with the pH values of Cullen, Keeler, and Robinson (9), the pH values of Hastings and Sendroy (4) (1924), the $p_{a_{H^+}}$ values of Hastings and Sendroy (12, 13) (1925, 1926) the $p_{a_{H^+}}$ values of Stadie and Hawes (14) (1928), but are not identical with the $p_{a_{H^+}}$ values of Sørensen and Linderstrøm-Lang. Their present value for $p_{a_{H^+}}$ is about 0.04 pH units higher than the pH values given here or the $p_{a_{H^+}}$ values of the above authors. All the pH values referred to are those based on the hydrogen electrode. While we agree with the suggestion of Sørensen and Linderstrøm-Lang that the problem of standardization should be treated according to the Lewis-Bjerrum activity theory, it appears better to accept Clark's suggestion (2) that the use of the term pH be continued. This avoids confusion in connecting new work with what has been done in the past. Hastings, Murray, and Sendroy (13) also emphasize the confusion in terms. The confusion that must be avoided is the *apparent* correction of values such as those of Cullen, Keeler, and Robinson by subtracting -0.04 when the difference is in the notation rather than in values.

RESULTS.

General Effects of Neutral Salt on pH of Buffer Solutions.

The influence of neutral salt on the hydrogen ion concentration of buffer solutions has been studied by many investigators (Michaelis and Krüger (15), Michaelis and Kakinuma (16), Leeper and Martin (17), Kolthoff and Bosch (18), Morton (19)).

The conclusions reached have been the same. The effect of a given concentration of salt is increased if the concentration of buffer is decreased. The effect is not the same in different buffer systems. Michaelis and Kakinuma find that the effect of NaCl upon the pH of 0.01 M acetic acid and 0.01 M sodium acetate is considerably less than Michaelis and Krüger found for phosphates.

The effect is much greater as the valence of the buffer increases. The cation effect seems to be the dominant factor in this change which increases in the order $K < Na < Li$. The present data show that these effects at 38° are similar to those found by the above

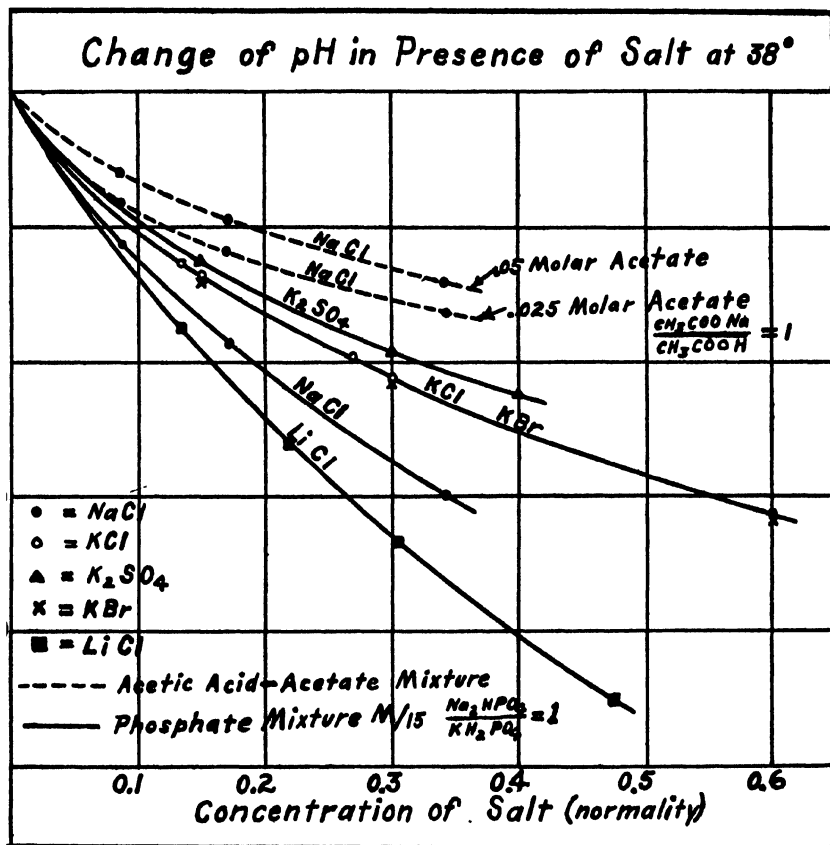


FIG. 1. Relative influence of various salts on the pH of $M/15$ phosphate buffers at 38° ; also comparative effect of NaCl on PO_4 and acetate systems.

authors in the neighborhood of 18° . Fig. 1 shows the relative reduction, ΔpH , in the pH due to the presence of sodium chloride in phosphate and acetic acid-acetate mixtures when the salt-acid ratio is 1:1, and also the comparative effects of sodium chloride,

potassium chloride, lithium chloride, potassium bromide, and potassium sulfate on the 1:1 phosphate mixture. The bromide and chloride effects are practically the same, which confirms what has been previously reported. Potassium sulfate cannot be called a true neutral salt and it is felt that its deviation is due to the secondary hydrolytic reaction $\text{SO}_4^{2-} + \text{H}^+ = \text{HSO}_4^-$. In line with this observation, Kolthoff and Bosch found that the effect of K_2SO_4 on the H^+ concentration in the carbonate-bicarbonate system was much less than that of KCl although the calculated ionic strength of the latter was only 0.5 and that of K_2SO_4 was 0.75, and Morton (19) also noted deviations with this salt.

Variation of pH Values with Salt-Acid Ratio and Concentration of Phosphate Buffers.

Summaries of the experiments on the effect of the changes in pH of phosphate solutions of various ratios of $\frac{\text{salt}}{\text{acid}}$ due to the presence of 1, 2, and 3 per cent of sodium chloride and potassium chloride are given in Tables I and II. The concentration of PO_4 was varied from $\text{M}/15$ to $\text{M}/60$. From Experiments 1 to 3 of Tables I and II it is evident that the effect of salt on the pH of a phosphate buffer containing the same ratios of di- and mono-salts is greater in the weaker phosphate solutions than in the stronger, also that the Δ pH for KCl is less than for NaCl . From the experiments of equal PO_4 concentrations but different salt-acid ratios, in Experiments 1, 4, and 6 of Table II, it is evident that the change in pH is not the same throughout the entire range of buffer. Thus in the $\text{M}/15$ PO_4 system, 1 per cent NaCl gives a Δ pH of 0.178 when the $\frac{\text{salt}}{\text{acid}}$ ratio is $\frac{8}{2}$ but a Δ pH of 0.220 when the $\frac{\text{salt}}{\text{acid}}$ ratio is $\frac{2}{8}$. This difference far exceeds experimental error. With each total PO_4 concentration the Δ pH increases as $\frac{\text{salt}}{\text{acid}}$ ratio decreases.

In analyzing these Δ pH values it was noticed that the Δ pH values for NaCl in a $\text{M}/15$ phosphate mixture containing a ratio of $\frac{\text{Na}_2\text{HPO}_4}{\text{KH}_2\text{PO}_4} = 0.25$ were approximately the same as that produced by the same amounts of salt in a $\text{M}/30$ phosphate solution con-

TABLE I.

Change in pH of Phosphate Solutions in Presence of NaCl.

Experi- ment No.	[PO ₄]	$\frac{[\text{Na}_2\text{HPO}_4]}{[\text{KH}_2\text{PO}_4]}$	Log <i>r</i>	[NaCl]	pH	Δ pH	pK'	Ionic strength.	$\sqrt{\mu}$
	<i>mols per l.</i>	<i>r</i>		<i>mols per l.</i>				μ	
1	0.0667	4	0.602	0	7.372		6.770	0.173	0.416
				0.086	7.267	0.105	6.665	0.258	0.508
				0.171	7.194	0.178	6.592	0.344	0.586
				0.342	7.090	0.282	6.488	0.515	0.717
2	0.0334	4	0.602	0	7.452		6.850	0.086	0.293
				0.086	7.312	0.140	6.710	0.171	0.413
				0.171	7.220	0.232	6.618	0.257	0.507
				0.342	7.100	0.352	6.498	0.428	0.654
3	0.0167	4	0.602	0	7.515		6.913	0.043	0.207
				0.086	7.335	0.180	6.733	0.128	0.358
				0.171	7.235	0.280	6.633	0.214	0.463
				0.342	7.100	0.415	6.498	0.385	0.620
4	0.0667	1	0.000	0	6.794		6.794	0.133	0.365
				0.086	6.682	0.112	6.682	0.219	0.468
				0.171	6.607	0.187	6.607	0.304	0.551
				0.342	6.495	0.299	6.495	0.475	0.689
5	0.0334	0.25	-0.602	0	6.307		6.909	0.046	0.214
				0.086	6.135	0.172	6.737	0.132	0.363
				0.171	6.045	0.262	6.647	0.217	0.466
				0.342	5.902	0.405	6.504	0.388	0.622
6	0.0667	0.25	-0.602	0	6.232		6.834	0.093	0.305
				0.086	6.096	0.136	6.698	0.178	0.422
				0.171	6.012	0.220	6.614	0.264	0.514
				0.342	5.890	0.342	6.492	0.435	0.659
7	0.0667	9	0.954	0	7.708		6.753	0.186	0.431
				0.086	7.611	0.097	6.657	0.271	0.520
				0.171	7.543	0.165	6.589	0.357	0.597
				0.342	7.434	0.274	6.480	0.528	0.726
8	0.0334	9	0.954	0	7.789		6.835	0.093	0.305
				0.086	7.651	0.138	6.697	0.178	0.422
				0.171	7.567	0.222	6.613	0.264	0.514
				0.342	7.449	0.340	6.495	0.435	0.659

TABLE I—*Concluded.*

Experiment No.	[PO ₄]	[Na ₂ HPO ₄] [KH ₂ PO ₄]	Log <i>r</i>	[NaCl]	pH	Δ pH	pK'	Ionic strength.	√ <i>μ</i>
	<i>mols per l.</i>	<i>r</i>		<i>mols per l.</i>				<i>μ</i>	
9	0.0667	7.48	0.874	0	7.633		6.759	0.184	0.429
				0.086	7.538	0.095	6.664	0.269	0.518
				0.171	7.468	0.165	6.595	0.355	0.596
				0.342	7.358	0.275	6.485	0.526	0.725
10	0.0667	6.69	0.826	0	7.581		6.755	0.183	0.427
				0.086	7.483	0.098	6.657	0.268	0.518
				0.171	7.411	0.170	6.585	0.354	0.594
				0.342	7.306	0.275	6.480	0.525	0.724
11	0.0667	2.33	0.368	0	7.142		6.774	0.160	0.400
				0.086	7.040	0.102	6.672	0.245	0.495
				0.171	6.964	0.178	6.596	0.331	0.575
				0.342	6.854	0.288	6.486	0.502	0.708

taining a ratio of $\frac{\text{Na}_2\text{HPO}_4}{\text{KH}_2\text{PO}_4} = 9$ (see Experiments 6 and 7, Table I). These mixtures have the same ionic strength.

"Ionic strength" is a concept introduced by Lewis and Randall (20) when studying activity coefficients in dilute solutions. They make the generalization that "in dilute solutions the activity coefficients of a given strong electrolyte are the same in all solutions of the same ionic strength." In the present paper the symbol μ , called ionic strength, is defined in mols per liter of solution instead of mols per 1000 gm. of water.

$$\mu = \frac{1}{2} (m_1 z_1^2 + m_2 z_2^2 + m_3 z_3^2 + \dots \text{etc.})$$

m_1, m_2, m_3, \dots = mols per liter of ions, A_1, A_2, A_3, \dots

z_1, z_2, z_3, \dots = valence of ions, A_1, A_2, A_3, \dots

This is the quantity that is employed in the Debye-Hückel equation. Disodium hydrogen phosphate and dihydrogen potassium phosphate are strong electrolytes and in the dilute solutions are assumed to be completely ionized. The basic salt gives rise to three ions B^+ , B^+ , and HPO_4^- , whereas the acid salt gives rise to two ions B^+ and H_2PO_4^- (when $\text{B} = \text{Na}$ or K). From the above relationship it can be calculated that the ionic strength of a

TABLE II.
Change in pH of Phosphate Solutions in Presence of KCl.

Experiment No.	[PO ₄]	$\frac{[Na_2HPO_4]}{[KH_2PO_4]}$	Log r	[KCl]	pH	Δ pH	pK'	Ionic strength.	$\sqrt{\mu}$
	<i>mols per l.</i>	<i>r</i>		<i>mols per l.</i>				μ	
1	0.0667	2.57	0.410	0	7.178		6.768	0.163	0.403
				0.067	7.112	0.066	6.702	0.229	0.479
				0.134	7.063	0.115	6.653	0.296	0.544
				0.268	6.994	0.184	6.584	0.431	0.656
2	0.0334	2.57	0.410	0	7.267		6.857	0.082	0.286
				0.067	7.163	0.104	6.753	0.148	0.385
				0.134	7.100	0.167	6.690	0.216	0.464
				0.268	7.017	0.250	6.607	0.350	0.591
3	0.0167	2.57	0.410	0	7.340		6.930	0.041	0.202
				0.067	7.196	0.144	6.786	0.108	0.328
				0.134	7.123	0.217	6.713	0.175	0.418
				0.268	7.030	0.310	6.620	0.309	0.556
4	0.0667	1.00	0.000	0	6.789		6.789	0.133	0.365
				0.067	6.710	0.079	6.710	0.200	0.447
				0.134	6.661	0.128	6.661	0.268	0.517
				0.268	6.591	0.197	6.591	0.402	0.634
5	0.0334	0.25	-0.602	0	6.304		6.906	0.046	0.214
				0.067	6.171	0.133	6.773	0.113	0.336
				0.134	6.097	0.207	6.699	0.180	0.425
				0.268	6.007	0.297	6.609	0.314	0.561
6	0.0667	0.25	-0.602	0	6.232		6.834	0.093	0.305
				0.067	6.137	0.095	6.739	0.160	0.400
				0.134	6.076	0.156	6.678	0.227	0.477
				0.268	5.996	0.236	6.598	0.361	0.601
7	0.0334	9.00	0.954	0	7.789		6.835	0.093	0.305
				0.067	7.693	0.096	6.739	0.160	0.400
				0.134	7.635	0.154	6.681	0.227	0.476
				0.268	7.555	0.234	6.601	0.361	0.600
8	0.0667	7.47	0.874	0	7.633		6.759	0.184	0.429
				0.067	7.570	0.063	6.696	0.251	0.501
				0.134	7.525	0.108	6.651	0.319	0.564
				0.268	7.455	0.178	6.581	0.453	0.673
9	0.0667	0.20	-0.698	0	6.127		6.825	0.089	0.298
				0.067	6.033	0.094	6.731	0.156	0.395
				0.134	5.973	0.154	6.671	0.223	0.472
				0.268	5.891	0.236	6.589	0.357	0.597

phosphate mixture is equal to the molal concentration of the uni-univalent BH_2PO_4 and 3 times that of the uni-bivalent B_2HPO_4 . A phosphate mixture consisting almost entirely of B_2HPO_4 will

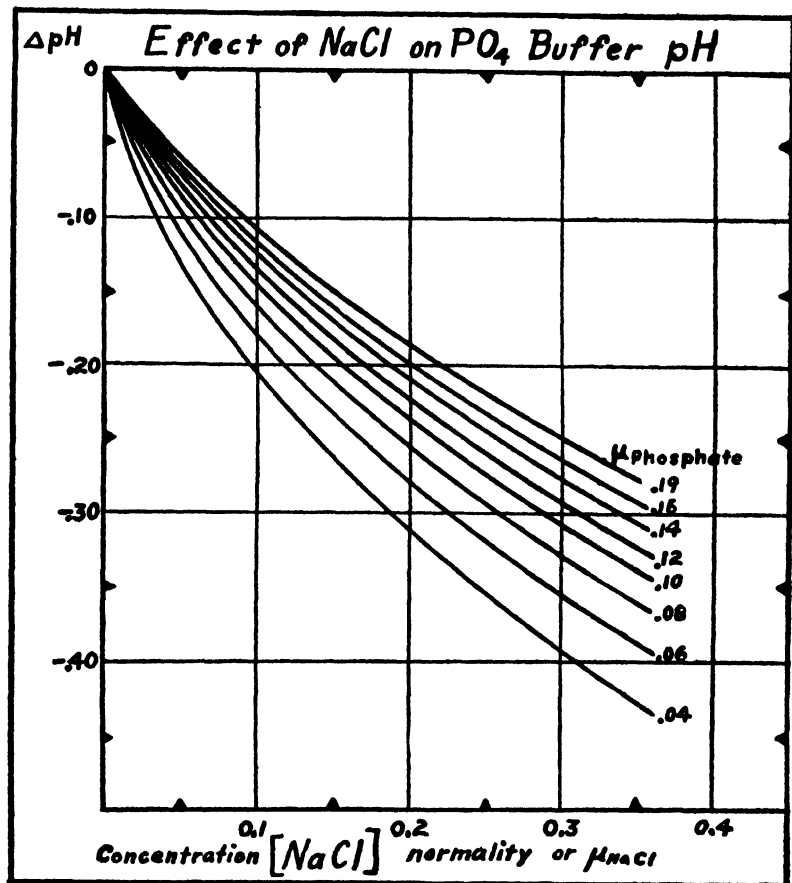


FIG. 2. Influence of μ of phosphate on NaCl effect on phosphate pH.

have a much higher ionic strength than a mixture that consists mostly of BH_2PO_4 although the total PO_4 concentration is the same. The changes in the concentrations of the ions HPO_4^- and H_2PO_4^- due to the change of the hydrogen ion concentration

($\text{HPO}_4^- + \text{H}^+ \rightleftharpoons \text{H}_2\text{PO}_4^-$) are negligible over the pH range studied here because the changes in H^+ are negligible in comparison to the concentration of phosphate ions. The last column in Tables I and II gives the square root of the ionic strength of the solutions.

It is clearly shown by the results that the change in pH due to the presence of a particular electrolyte in a phosphate mixture is

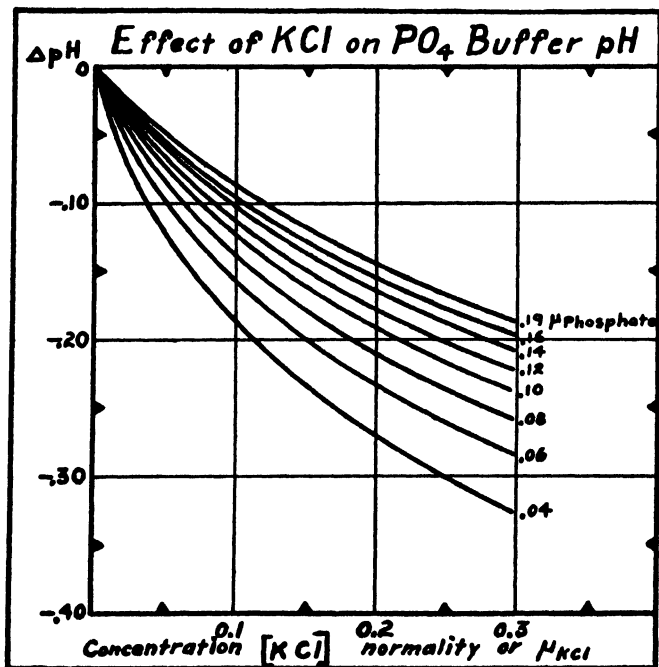


FIG. 3. Influence of μ of phosphate on KCl effect on phosphate pH.

related to the ionic strength of the buffer solution and not directly to the total PO_4 concentration. The ΔpH varies over the entire range of the buffer because the ionic strength varies with the ratio of $\frac{\text{uni-bivalent } \text{B}_2\text{HPO}_4}{\text{uni-univalent } \text{BH}_2\text{PO}_4}$.

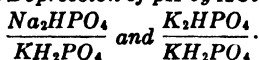
Figs. 2 and 3 show the ΔpH effects of NaCl and KCl when present up to concentrations of 0.3 N in solutions of phosphate mixtures of known ionic strength.

Interchangeability of K and Na in the Sørensen PO₄ System.

In the above experiments with the Sørensen PO₄ system, two cations, sodium and potassium, are present. It was necessary to see whether the effects were different when only one cation was present. The experiments were repeated with K₂HPO₄-KH₂PO₄ mixtures. The latter was made by adding standardized potassium hydroxide to the KH₂PO₄, so that the total concentration of PO₄ was known. In Table III a typical experiment is given.

TABLE III.

Experiment Showing That Depression of pH by KCl Is the Same in the Systems



Total concentration of PO₄ in both cases is 0.03334 M.

Solution A = mixture of Na₂HPO₄ and K₂HPO₄.

" B = " " K₂HPO₄ + KOH.

Solution A.			Solution B.		
[KCl]	pH	Δ pH	[KCl]	pH	Δ pH
<i>N</i>			<i>N</i>		
0	7.789		0	7.727	
0.067	7.693	0.096	0.067	7.632	0.095
0.171	7.635	0.154	0.171	7.573	0.154
0.342	7.555	0.234	0.342	7.493	0.234

This experiment is given because it represents the condition at higher pH, when there is the greater proportion of Na. • From the results the differences in Δ pH obtained from the presence of KCl in the K₂HPO₄-KH₂PO₄ mixtures are, within experimental error, the same as those obtained in the Na₂HPO₄-KH₂PO₄ mixtures.

DISCUSSION.

Effect of Salt on the Second pK' of Phosphoric Acid.

About 20 years ago Henderson (21) and Washburn (22) simultaneously characterized the equilibrium of a buffer solution in terms of the law of mass action, assuming that the concentration of the negative ion approached the total concentration of salt and that the concentration of the undissociated acid was equal to the total concentration of the acid. The general expression is

$$[H^+] = K' \frac{[\text{salt}]}{[\text{acid}]}$$

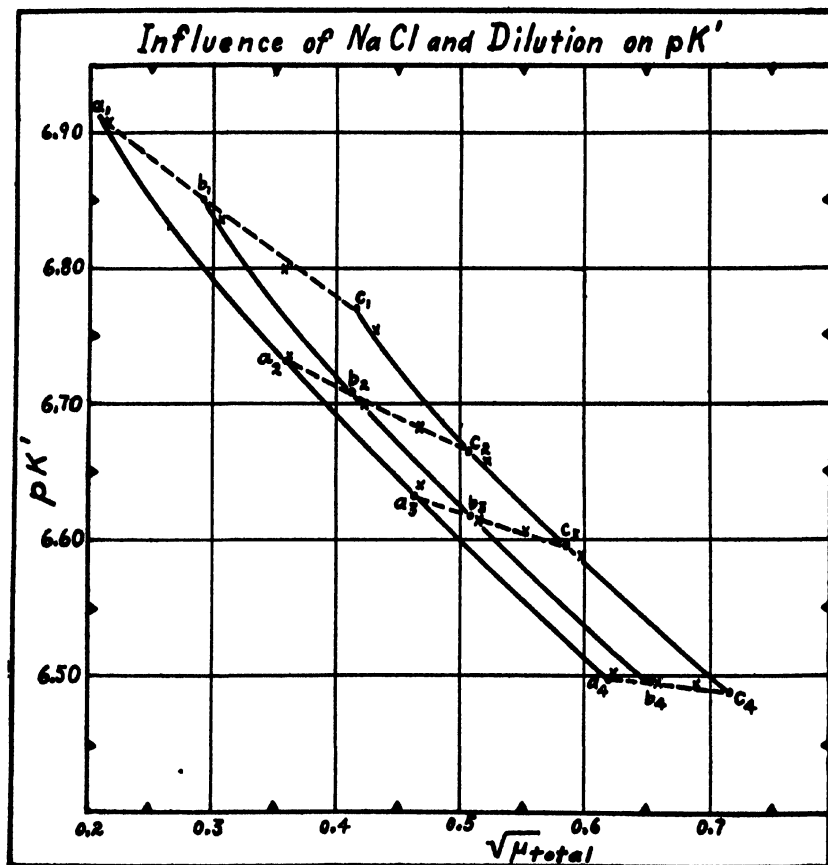


FIG. 4.

a_1, a_2, a_3, a_4 , $m/60$ PO_4 containing 0, 0.5, 1, 2 per cent NaCl respectively.

b_1, b_2, b_3, b_4 , $m/30$ " " 0, 0.5, 1, 2 " " " "

c_1, c_2, c_3, c_4 , $m/15$ " " 0, 0.5, 1, 2 " " " "

Line $c_1b_1a_1$, dilution with water of $m/15$ PO_4 to $m/30$ and $m/60$.

a_2, b_2, c_2 , $m/60$, $m/30$, $m/15$ PO_4 containing 0.5 per cent (0.086 N) NaCl.

a_3, b_3, c_3 , $m/60$, $m/30$, $m/15$ " " 1.0 " " (0.172 " " "

a_4, b_4, c_4 , $m/60$, $m/30$, $m/15$ " " 2.0 " " (0.344 " " "

in which the bracketed expressions represent the concentration of hydrogen ion, weak acid, and the salt of the weak acid and K' is the proportionality constant, called the apparent dissociation constant, which approaches the dissociation constant, K , with dilution.

With the general use of Sørensen's term pH, Hasselbalch first used the above relationship in its logarithmic form, $\text{pH} = \text{pK}' + \log \frac{[\text{salt}]}{[\text{acid}]}$. This equation applied to dilute phosphate mixture is only an approximation. Michaelis and Krüger showed that on dilution of a phosphate mixture with water the reaction became more alkaline. This would not occur if the reaction were only due to the ratio of the constituents and the pK' remained constant. Cohn (23) recalculated Sørensen's E.M.F. measurements of phosphate mixtures and shows that the variation in pK' , while small in comparison with the change in pH, is 10 times greater than the probable error. The pK' changes with the ionic strength of the solution and will approach a true pK value at infinite dilution when the phosphate mixture obeys the simple laws of an ideal solution.

With phosphate mixtures this change of pK' with the ionic strength of the solution is not a straight line. This is observed when the data of Table I or Table II are plotted in a graph. This fact also is shown over a wider range of concentration in Fig. 2 of Cohn's paper. When neutral salt is added to the phosphate buffer system, the curves showing change in pK' are distinctly different for varying quantities of salt. pK' values are lower and the curves flatter with increase of salt concentration. This is shown in Fig. 4 where the pK' values of Experiments 1 to 3 of Table I are plotted against μ_{total} .

The line $c_1c_2c_3c_4$ (Fig. 4) represents the change in pK' with ionic strength in a M/15 phosphate mixture due to the presence of sodium chloride. The line $c_1b_1a_1$ represents the change of pK' with ionic strength by dilution of the same M/15 phosphate mixture up to M/60 with water. The same curve would have been followed if the ionic strength of the M/15 phosphate mixtures had been changed by changing the ratio of basic salt to acid salt. The x points are points taken from other experiments in Table I. The points a_2 , b_2 , and c_2 represent M/60, M/30, and M/15 respectively,

containing 0.5 per cent (0.086 N) NaCl, a_3 , b_3 , and c_3 represent the same phosphates containing 1 per cent (0.171 N) NaCl and a_4 , b_4 , and c_4 represent the same phosphates containing 2 per cent (0.342 N) NaCl. The presence of salt flattens the curves of the change of pK' with ionic strength.

Another fact brought out by Fig. 4 is the stabilizing action on the pH of a buffer solution containing neutral salt when the solution is diluted with the neutral salt solution. If we start at c_4 (Fig. 4) we have a M/15 phosphate solution containing 2 per cent (0.342 N) sodium chloride. If this solution is diluted once with water, the PO_4 concentration becomes M/30 and the sodium chloride 0.171 N, which on the curve is point b_3 . The pH is 0.12 more alkaline. If, however, the dilution had been made with a 2 per cent (0.342 N) sodium chloride solution, the point b_4 is reached and the change in pH would have been only about 0.005. Likewise in diluting this same M/15 phosphate + NaCl (c_4) solution to M/60 with water, the change in pH is 0.245 (c_4 to a_2), whereas the dilution with 2 per cent sodium chloride makes a change of only 0.01 pH (c_4 to a_4).

*Application of Debye-Hückel Equation to Phosphate Buffers
Containing Neutral Salt.*

Cohn (23) has studied the phosphate buffer solutions according to the modern conception of activity and has been able to account for the deviations by means of the Debye-Hückel equation, which assumes that in completely ionized strong electrolytes the deviation from the ideal law of mass action in dilute solutions is caused mainly by the interionic electrical effects.

The mass law equation for the second dissociation constant for phosphoric acid may be written

$$\frac{a_{H^+} a_{HPO_4^{2-}}}{a_{H_2PO_4^-}} = K \quad (1)$$

where a_{H^+} = activity of H^+ , $a_{HPO_4^{2-}}$ = activity of HPO_4^{2-} , and $a_{H_2PO_4^-}$ = activity of $H_2PO_4^-$; or in logarithmic form

$$pH - \log \frac{a_{HPO_4^{2-}}}{a_{H_2PO_4^-}} = pK \quad (2)$$

The activity coefficient γ is defined as the factor by which the concentration must be multiplied to give the activity.

Substituting for $a_{\text{HPO}_4^-}$ its equivalent $[\text{HPO}_4^-] \gamma_2$ and for $a_{\text{H}_2\text{PO}_4^-}$ its equivalent $[\text{H}_2\text{PO}_4^-] \gamma_1$ where $[\text{HPO}_4^-]$ and $[\text{H}_2\text{PO}_4^-]$ signify concentrations of these ions and γ_2 and γ_1 are the activity coefficients of the HPO_4^- and H_2PO_4^- respectively, the following equation is obtained

$$\text{pH} - \log \frac{[\text{HPO}_4^-]}{[\text{H}_2\text{PO}_4^-]} + \log \frac{\gamma_1}{\gamma_2} = \text{pK} \quad (3)$$

Assuming complete ionization of the phosphate salts, the total concentrations of the salts may be substituted for the concentration of the ions.

$$[\text{H}_2\text{PO}_4^-] = [\text{KH}_2\text{PO}_4] \text{ and } [\text{HPO}_4^-] = [\text{Na}_2\text{HPO}_4]$$

therefore

$$\text{pH} = \text{pK} + \log \frac{[\text{Na}_2\text{HPO}_4]}{[\text{KH}_2\text{PO}_4]} - \log \frac{\gamma_1}{\gamma_2} \quad (4)$$

According to the theory of Debye and Hückel, in solutions which are not too concentrated the activity coefficient γ of an ion may be defined in the following manner

$$-\log \gamma = \frac{0.5 z^2 \sqrt{\mu}}{1 + 3.29 \times 10^7 \times a \sqrt{\mu}}$$

where

z = valence of the ion

μ = ionic strength of solution

a = average effective diameter of all the ions

The constants 0.5 and 3.29×10^{-7} are only approximations, involving variables that may change slightly in different media. In Table 66 (p. 500) of Clark (2) it is shown that the effect of temperature is practically negligible on these constants.

$$-\log \gamma_1 = \frac{0.5 \sqrt{\mu}}{1 + 3.29 \times 10^7 \times a \sqrt{\mu}}$$

TABLE IV.—Application of Debye-Hückel Equation to Phos

Experiment No.	pK'	$\sqrt{\mu}$	$\frac{\gamma_1}{\gamma_2}$ (pK = 7.15)	$\frac{1.5\sqrt{\mu}}{1 + 1.5\sqrt{\mu}}$	ΔY^* (3) - (4)	pK'	$\sqrt{\mu}$	$\frac{\gamma_1}{\gamma_2}$ (pK = 7.15)	$\frac{1.5\sqrt{\mu}}{1 + 1.5\sqrt{\mu}}$	ΔY^* (8) - (9)	Deviation from Average
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	
Without salt.					0.086 N NaCl.						
1	6.770	0.416	0.380	0.384	-0.004	6.665	0.508	0.485	0.432	0.053	0.0
2	6.850	0.293	0.300	0.305	-0.005	6.710	0.413	0.440	0.383	0.057	0.0
3	6.913	0.207	0.237	0.237	0.000	6.733	0.358	0.417	0.349	0.068	-0.01
4	6.794	0.365	0.356	0.354	+0.002	6.682	0.468	0.468	0.412	0.056	0.0
5	6.834	0.305	0.316	0.314	+0.002	6.698	0.422	0.452	0.388	0.064	-0.0
6	6.909	0.214	0.241	0.243	-0.002	6.737	0.363	0.413	0.353	0.060	-0.0
7	6.753	0.431	0.397	0.393	+0.004	6.657	0.520	0.493	0.438	0.055	0.0
8	6.835	0.305	0.315	0.314	+0.001	6.697	0.422	0.453	0.388	0.065	-0.0
9	6.759	0.429	0.391	0.391	0.000	6.664	0.518	0.486	0.437	0.049	0.0
10	6.755	0.427	0.395	0.390	+0.005	6.657	0.518	0.493	0.437	0.056	0.0
11	6.774	0.400	0.376	0.375	+0.001	6.672	0.495	0.478	0.426	0.052	0.0
Average.....										0.058	
					0.067 N KCl.						
1	6.768	0.403	0.382	0.373	0.009	6.702	0.479	0.448	0.418	0.030	0.0
2	6.857	0.286	0.293	0.300	-0.007	6.753	0.385	0.397	0.366	0.031	0.0
3	6.930	0.202	0.220	0.232	-0.012	6.786	0.328	0.364	0.330	0.034	0.0
4	6.789	0.365	0.361	0.354	0.007	6.710	0.447	0.440	0.401	0.039	-0.0
5	6.906	0.214	0.244	0.243	0.001	6.773	0.336	0.377	0.335	0.042	-0.0
6	6.834	0.305	0.316	0.314	0.002	6.739	0.400	0.411	0.375	0.036	0.0
7	6.835	0.305	0.315	0.314	0.001	6.739	0.400	0.411	0.375	0.036	0.0
8	6.759	0.429	0.391	0.391	0.000	6.696	0.501	0.454	0.429	0.025	0.0
9	6.825	0.298	0.325	0.309	-0.016	6.731	0.395	0.419	0.372	0.047	-0.0
Average.....										0.035	

* ΔY (see p. 795) is the discrepancy between the observed and calculated value

Buffer Systems. Effect of Presence of NaCl and KCl.

pK'	$\sqrt{\mu}$	$\frac{\gamma_1}{\gamma_2}$ ($pK = 7.15$)	$\frac{1.5\sqrt{\mu}}{1 + 1.5\sqrt{\mu}}$	ΔY^* (14) - (15)	Deviation from average.	pK'	$\sqrt{\mu}$	$\frac{\gamma_1}{\gamma_2}$ ($pK = 7.15$)	$\frac{1.5\sqrt{\mu}}{1 + 1.5\sqrt{\mu}}$	ΔY^* (20) - (21)	Deviation from average.
(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)
0.171 N NaCl.						0.342 N NaCl.					
5.592	0.586	0.558	0.468	0.090	0.004	6.488	0.717	0.662	0.518	0.144	0.009
6.618	0.507	0.532	0.432	0.100	-0.006	6.498	0.654	0.652	0.495	0.157	-0.004
6.633	0.463	0.517	0.410	0.107	-0.013	6.498	0.620	0.652	0.482	0.170	-0.017
6.607	0.551	0.543	0.453	0.090	0.004	6.495	0.689	0.655	0.508	0.147	0.006
6.614	0.514	0.536	0.435	0.101	-0.007	6.492	0.659	0.658	0.497	0.161	-0.008
6.647	0.466	0.503	0.411	0.092	0.002	6.514	0.622	0.636	0.483	0.153	0.000
6.589	0.597	0.561	0.472	0.089	0.005	6.480	0.726	0.670	0.521	0.149	0.004
6.613	0.514	0.537	0.435	0.102	-0.008	6.495	0.659	0.655	0.497	0.158	-0.005
6.595	0.596	0.555	0.472	0.083	0.011	6.485	0.725	0.665	0.521	0.144	0.009
6.585	0.594	0.565	0.471	0.094	0.000	6.480	0.724	0.670	0.521	0.149	0.004
6.596	0.575	0.554	0.463	0.091	0.003	6.486	0.708	0.664	0.515	0.149	0.004
				0.094						0.153	
0.134 N KCl.						0.268 N KCl.					
6.653	0.544	0.497	0.449	0.048	0.004	6.584	0.656	0.566	0.496	0.070	0.006
6.690	0.464	0.460	0.410	0.050	0.002	6.607	0.591	0.543	0.470	0.073	0.003
6.713	0.418	0.437	0.385	0.052	0.000	6.620	0.556	0.530	0.455	0.075	0.001
6.661	0.517	0.489	0.437	0.052	0.000	6.591	0.634	0.559	0.487	0.072	0.004
6.699	0.425	0.451	0.389	0.062	0.010	6.609	0.561	0.541	0.457	0.084	0.008
6.678	0.477	0.472	0.417	0.055	0.003	6.598	0.601	0.552	0.474	0.078	0.002
6.681	0.476	0.469	0.417	0.052	0.000	6.601	0.600	0.549	0.474	0.075	0.001
6.651	0.564	0.499	0.458	0.041	0.011	6.581	0.673	0.569	0.502	0.067	0.009
6.671	0.472	0.479	0.415	0.064	0.012	6.589	0.597	0.561	0.472	0.089	0.013
				0.052						0.076	

or the Debye-Hückel correction term.

$$\begin{aligned}
 -\log \gamma_2 &= \frac{2 \sqrt{\mu}}{1 + 3.29 \times 10^7 \times a \sqrt{\mu}} \\
 -\log \gamma_2 - (-\log \gamma_1) &= \frac{1.5 \sqrt{\mu}}{1 + 3.29 \times 10^7 \times a \sqrt{\mu}} \\
 \log \frac{\gamma_1}{\gamma_2} &= \frac{1.5 \sqrt{\mu}}{1 + 3.29 \times 10^7 \times a \sqrt{\mu}}
 \end{aligned}$$

With an a value of 4.56×10^{-8} , whence

$$\log \frac{\gamma_1}{\gamma_2} = \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} \quad (5)$$

and a pK of 7.16, Cohn found that Equation 4 described Sørensen's phosphates with an accuracy of ± 0.01 pH.

Kolthoff (24) in studying the influence of dilution on the pH of buffer solutions used practically the same equation for $\log \frac{\gamma_1}{\gamma_2}$ except that he gave to a a slightly lower value (4×10^{-8}).

Cohn's equation was applied to the present data with a slight correction of the pK value to take care of the temperature difference. He used the Sørensen standardization at 18° which would give a value for pK 0.01 unit lower than that which would be obtained by the Cullen method of standardization at 20° . The pK at 38° according to the latter standardization would be approximately 0.03 units less than the value at 20° . This would give a value of 7.145 which is in agreement with the value of 7.15 estimated by Hastings, Murray, and Sendroy. With this value of 7.15, and with the pH determined, the values for $\log \frac{\gamma_1}{\gamma_2}$ given in Column 3, Table IV are obtained thus:

$$\log \frac{\gamma_1}{\gamma_2} = 7.15 - \left(\text{pH} - \log \frac{[\text{Na}_2\text{HPO}_4]}{[\text{KH}_2\text{PO}_4]} \right) \quad (6)$$

By using the value for the average effective diameter of the ions as given by Cohn, it is found that for the range of phosphate studied ($M/60$ to $M/15$) the values calculated for $\log \frac{\gamma_1}{\gamma_2}$ by Equation

5, given in Column 4, Table IV, agree with the determined value to within 0.01 pH at 38°. The present data for pure phosphate at 38° are in agreement with Cohn's finding for 18°.

However, when the same comparison between observed values for $\log \frac{\gamma_1}{\gamma_2}$ (Columns 8, 14, 20) and calculated values (Columns 9, 15, 21) is made for phosphate mixture containing NaCl or KCl, it is found that the calculated and observed values no longer agree. The discrepancies, designated by ΔY are given in Columns 10, 16, and 22. This lack of agreement does not prove entirely that the assumptions made in using this term are wrong, or that the presence of sodium or potassium chloride has more than an inter-ionic effect on the activity of the phosphate ions. Without doubt an error was made in assuming that the average effective diameter of all the ions present in the solution remained the same in the presence of sodium or potassium ions. Hückel (7) calculated a value for the average effective diameter for potassium chloride equal to 3.76×10^{-8} and for sodium chloride equal to 2.35×10^{-8} . These values are smaller than 4.56×10^{-8} which was used in the correction term. A smaller value for a would decrease the difference existing between $\log \frac{\gamma_1}{\gamma_2}$ and the term $\frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}}$. In the present paper, because of the uncertainty of these magnitudes, no attempt to adjust this value of a is made.

From the data it was striking that the differences ΔY between $\log \frac{\gamma_1}{\gamma_2}$ and $\frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}}$ for a given concentration of salt approached a constant over varying ranges of PO_4 concentration, but that it varied as a definite function of the neutral salt concentration. In most cases the variation of this value for a given salt concentration was within 0.01 units of the average mean value for that concentration. This quantity therefore may be used as a correction factor when one has a known phosphate buffer containing a known amount of sodium or potassium chloride respectively. The equation then will be

$$\text{pH} = 7.15 + \log \frac{[\text{Na}_2\text{HPO}_4]}{[\text{KH}_2\text{PO}_4]} - \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} - \Delta Y \quad (7)$$

The values for ΔY for potassium and sodium chlorides in concentrations up to 0.3 N may be obtained from Fig. 5.

When ΔY is plotted against the square root of the concentration of the neutral salt (in uni-univalent salt this is equivalent to the square root of ionic strength of the salt) it is found that the values fall on a straight line (lower curves of Fig. 5). The slopes of the lines are 0.333 and 0.150 for sodium chloride and potassium

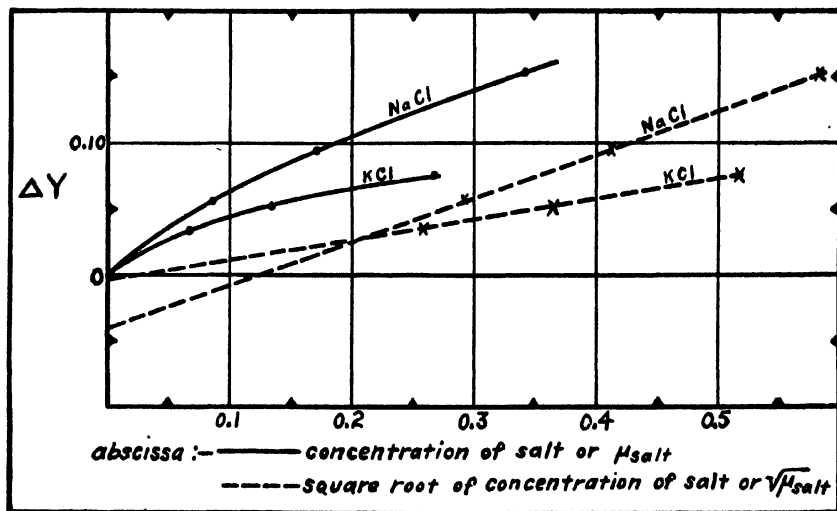


FIG. 5. Deviations from the Debye-Hückel equation ΔY due to the presence of NaCl and KCl.

chloride respectively, and the lines cross the ordinate at points -0.04 and at 0 .

The equations of this correction are

$$\Delta Y_{\text{NaCl}} = 0.333 \sqrt{\mu_{\text{NaCl}}} - 0.04$$

$$\Delta Y_{\text{KCl}} = 0.150 \sqrt{\mu_{\text{KCl}}}$$

where μ_{NaCl} and μ_{KCl} equal the ionic strengths of the sodium and potassium chloride in the solution.

When these values for ΔY are introduced the equations are:

For sodium chloride

$$\text{pH} = 7.15 + \log \frac{[\text{Na}_2\text{HPO}_4]}{[\text{KH}_2\text{PO}_4]} - \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} - (0.333 \sqrt{\mu_{\text{NaCl}}} - 0.04) \quad (8)$$

For potassium chloride

$$\text{pH} = 7.15 + \log \frac{\text{Na}_2\text{HPO}_4}{\text{KH}_2\text{PO}_4} - \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} - (0.150 \sqrt{\mu_{\text{KCl}}}) \quad (9)$$

These equations are valid for calculating the pH to ± 0.02 unit of a known phosphate mixture containing a known concentration (up to 0.50 N) of sodium or potassium chloride.

Practical Application of Factors for Dilution and Salt Effect.

It is general practice in biological work to use phosphate buffer at other than M/15 concentration with the assumption that the pH is that which the $\frac{\text{salt}}{\text{acid}}$ ratio used gives in M/15 concentration; i.e., the Sørensen value for M/15 PO_4 . This is incorrect since the pH changes with the concentration and is, as shown above, a function of the ionic strength. The same mistake is commonly made when for injection and for bacteriological work the PO_4 is prepared in isotonic saline (physiologically normal) (0.9 to 0.85 per cent). The actual pH here (see Fig. 4) is decreased. Another practice is to use a low buffer concentration (e.g., M/50) in isotonic saline. In this case the actual pH is dependent upon the algebraic sum of the two errors, increase due to dilution and decrease due to presence of salt.

To correct for concentration, whenever it is desirable to know or to establish the pH with an error not greater than 0.02 pH, it is necessary to correct for both the buffer concentration and the salt concentration. This is shown clearly in the analysis of Fig. 4 above.

The actual pH may be obtained by use of Equations 8 or 9, or it may be obtained to within 0.02 pH graphically with the aid of Figs. 2 and 6. Fig. 2 gives the effect, in terms of Δ pH, of NaCl concentration between 0 and 0.4 N on various PO_4 concentrations (given here as a series of curves with varying ionic strength, μ). Fig. 6 is constructed for two purposes; first, to give μ for the usual concentrations and $\frac{\text{salt}}{\text{acid}}$ ratios of PO_4 buffers, and second, to give the value for the term $\log \frac{\gamma_1}{\gamma_2}$ of Equation 4 for any μ .

The difference between the values of $\log \frac{\gamma_1}{\gamma_2}$ for any two different

PO_4 concentrations *with the same ratio* represents the change in pH due to change in concentration. This is evident from the following consideration.

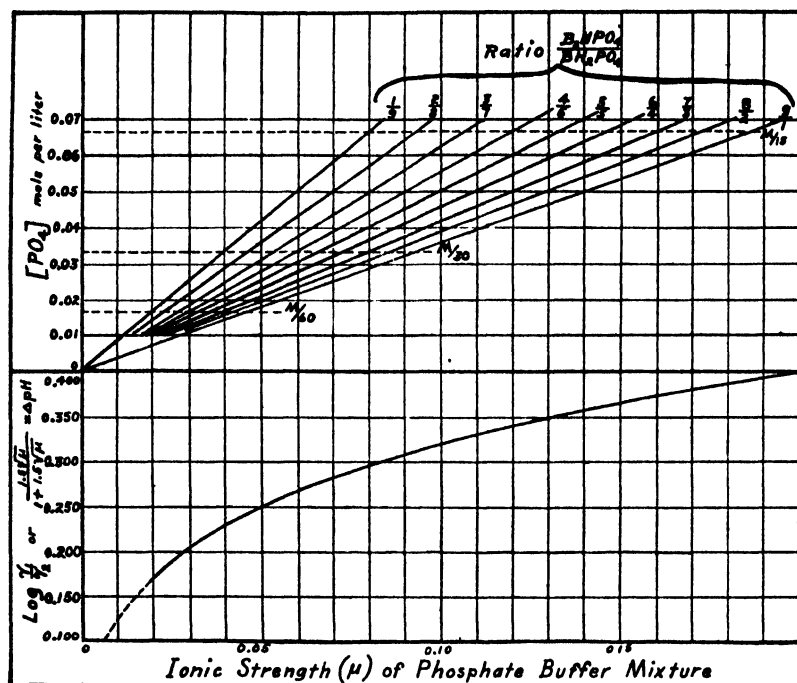


FIG. 6. The upper half represents the relation between molar concentration of phosphate, of $\frac{\text{salt}}{\text{acid}}$ ratio, and ionic strength of phosphate solution.

The lower half gives the curve for the term $\log \frac{\gamma_1}{\gamma_2} \left(= \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} \right)$ in the equation $\text{pH} = 7.15 + \log r - \log \frac{\gamma_1}{\gamma_2}$. The difference in ordinates, therefore, of the lower half for any two PO_4 concentrations with the same ratio represents the ΔpH due to the change in concentration. The dotted lines show the usual $m/15$ concentration and the two- and fourfold dilutions $m/30$ and $m/60$ used often in this work.

Thus, if the initial pH_1 and μ_1 are known and it is desired to know the final pH_2 after dilution to a concentration μ_2

$$\text{pH}_1 = \text{pK} + \log r - \log \left(\frac{\gamma_1}{\gamma_2} \right)_1 \quad \text{and} \quad \text{pH}_2 = \text{pK} + \log r - \log \left(\frac{\gamma_1}{\gamma_2} \right)_2$$

then

$$\text{pH}_2 = \text{pH}_1 + \log \left(\frac{\gamma_1}{\gamma_2} \right)_1 - \log \left(\frac{\gamma_1}{\gamma_2} \right)_2 \quad \text{and} \quad \Delta \text{pH} = \log \left(\frac{\gamma_1}{\gamma_2} \right)_1 - \log \left(\frac{\gamma_1}{\gamma_2} \right)_2$$

With dilution ΔpH is positive.

The usefulness of Figs. 2 and 6 is best shown by numerical examples.

Case 1. Effect of Dilution.—If $\text{m}/15 \text{ PO}_4$, with a ratio $\frac{8}{2}$ and $\text{pH } 7.372$ is to be diluted to $\text{m}/30$, the pH may be calculated thus: From Fig 6. the ionic strength changes from 0.1733 to 0.0860 and $\log \frac{\gamma_1}{\gamma_2}$ changes from 0.384 to 0.305 . $\Delta \text{pH} = 0.384 - 0.205 = 0.079$. $\text{pH } \text{m}/30 = 7.372 + 0.079 = 7.451$. Determined electrometrically, $\text{pH} = 7.452$.

Case 2. Effect of Sodium Chloride.—If $\text{m}/30 \text{ PO}_4$, ratio $\frac{8}{2}$, is prepared in 0.9 per cent NaCl (normal saline for injection) the resultant pH may be calculated as follows:

(a) $\text{m}/15$ diluted to $\text{m}/30$ gives $\Delta \text{pH} = +0.079$ as above.

(b) 0.9 per cent $\text{NaCl} = 0.154 \text{ N}$. From Fig. 2, read that 0.154 N NaCl in PO_4 solution with μ of 0.086 gives a ΔpH of -0.22 .

The pH of $\text{m}/30 \text{ PO}_4$, ratio $\frac{8}{2}$ in 0.9 NaCl is, therefore, $7.372 + 0.079 - 0.22 = 7.229$.

Case 3. Dilution at Constant pH.—In some cases, it is desirable to dilute a buffer mixture without change of pH . In Case 1 dilution of $\text{m}/15$ to $\text{m}/30$ results in an increase of pH of 0.08 . By reading from Fig. 2 the point where the ΔpH of 0.08 intersects the curve for μ of 0.086 , one finds a NaCl concentration of 0.035 N . The addition of salt to this concentration, or in this case the use of 0.070 N NaCl as diluting agent, would have stabilized the pH at the initial pH .

So far we have been able to solve this type of problem only by the graphic method. The examples of Cases 1 and 2 above can be easily solved by Equation 8..

Applicability to Buffer Index Values.

Another condition in which these corrections may be important has been suggested by Morton's discussion of buffer solution.

Morton suggests that Van Slyke's (25) buffer index $\beta = \frac{dB}{dpH}$ should be supplemented by a unit $\pi = \frac{dpH}{d\sqrt{\mu}}$ to define the stability of the buffer system against dilution.

When β is determined experimentally or calculated from an actual titration curve, it must include this dilution factor; but when it is calculated from the dissociation constants and $\frac{\text{salt}}{\text{acid}}$ ratio of the buffer, the change in pH due either to dilution or to the effect of neutral salt formed should be considered. The need for this correction when necessary being granted, it seems much more desirable to define a buffer in terms of Van Slyke's buffer index $\beta = \frac{dB}{dpH}$ than to use two independent criteria.

CONCLUSIONS.

From the experimental data given above, and from the equations deduced from them, it is felt that the conditions existing in phosphate buffers, either without or with the addition of neutral salts, can be understood more clearly than hitherto. The results reported here for 38° agree well with others for room temperature and indicate that the temperature effect on the Debye-Hückel constant is negligible. The fact that in mixtures of phosphate and alkali halides it is necessary to consider the ionic concentration of the neutral salt separately rather than the total ionic concentration is interesting. It is probable that some adjustment of the constants may make this unnecessary, but since adjustment of constants is empirical, it seemed best for the present to separate the effects. The accuracy with which this treatment represents the experimental data should justify itself for the major purpose of this work, which was to obtain information concerning this system which could be used in biological work.

With this object, Fig. 6 has been worked out to make the information readily available to biologists in general. It is hoped eventually to express the data in nomogram form.

SUMMARY.

1. The influence of neutral salts on pH has been studied throughout the entire range of the phosphate buffer solution. The effect

is predominantly that of the cations, and increases in the order K, Na, Li.

2. The effect of neutral salt is independent of the *total* PO_4 concentration, but is a function of the ionic strength. This varies over the buffer range because of the change in proportions of the salts, B_2HPO_4 and BH_2PO_4 , which ionize into three and two ions respectively.

3. The dilution of buffer solutions with solutions of neutral salts rather than water, in order to minimize the change in ionic strength, has been discussed as a method of stabilizing the pH.

4. By adjusting Cohn's pK for the second dissociation constant of phosphoric acid to 38° and to the Cullen standardization, the equation of Cohn

$$\text{pH} = \text{pK} + \log \frac{\text{Na}_2\text{HPO}_4}{\text{KH}_2\text{PO}_4} - \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}}$$

is found to be valid at 38° for concentrations of PO_4 up to $\text{m}/15$ within the error of ± 0.01 pH.

5. This equation, when applied to phosphate mixtures containing neutral salts, is not valid, but may be corrected by an added term which is a function of the ionic strength of neutral salt only.

6. The application of these data to biological practice is discussed and a graphic method of calculation is given.

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